THE CHINESE UNIVERSITY OF HONG KONG

CELLULOLYTIC AND HEMICELLULOLYTIC ENZYMES OF *FLAMMULINA VELUTIPES*

A THESIS SUBMITTED TO
THE DIVISION OF BIOLOGY, GRADUATE SCHOOL
AS PARTIAL FULFILLMENT FOR THE DEGREE OF
MASTER OF PHILOSOPHY

DEPARTMENT OF BIOLOGY

BY

CHEUNG PUI YI

SHATIN, NEW TERRITORIES, HONG KONG
JULY 1994

UL

Abstract

Flammulina velutipes, also called the "winter mushroom", was first cultivated in China around A.D. 800-900. In 1986, it ranked sixth in the total worldwide production of edible mushrooms. In spite of its economic importance, little is known about the ability of the fungus to degrade the cellulose, hemicellulose and lignin components of the substrates used for growth. In the present study, the fungus was grown in submerged culture using purified components of lignocellulose or natural lignocellulosic substrates as the major sources of carbon, and the production of cellulolytic (exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, ß-glucosidase) and hemicellulolytic (endo-1,4-ß-xylanase, ß-xylosidase) enzymes was examined.

Preliminary studies on the lignocellulolytic enzyme profile of *F. velutipes* grown on Avicel and birchwood xylan revealed that this fungus produced detectable levels of exoglucanase (Avicelase), endoglucanase (carboxymethylcellulase), extracellular and cell-associated ß-glucosidase, ß-xylanase, and extracellular and cell-associated ß-xylosidase. Optimal temperatures and optimal pH values for the activity of the enzymes were determined.

Time course experiments in which enzyme production profiles were determined following fungal growth on Avicel or birchwood xylan indicated that *F. velutipes* was highly xylanolytic but only moderately cellulolytic. Enzyme induction patterns observed after transferring fungal biomass grown on Potato Dextrose Broth (PDB) to media containing different substrates for enzyme production (Avicel, carboxymethylcellulose, filter paper, cotton wool, rice straw and birchwood xylan) revealed that rice straw was the best substrate for the production of both cellulolytic and xylanolytic enzymes.

Partial purification of cellulase proteins produced by *F. velutipes* grown on Avicel-supplemented medium indicated that the fungus

synthesized at least one exoglucanase, two endoglucanases and one extracellular ß-glucosidase. Results deduced from enzyme assays and activity staining on Native-PAGE suggested that at least 2 protein bands separated by Native-PAGE exhibited more than one cellulolytic activity.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. J. A. Buswell, for his continuous guidance and invaluable discussions throughout the entire period of study. I also thank him for his patience in introducing me to the field of mushroom enzymology and careful review of this thesis.

I should be grateful to Dr. H. S. Kwan and Dr. K. H. Yung for reading the manuscript and providing valuable comments on this thesis. I must also thank Professor A. J. P. Trinci for acting as the external examiner of my thesis.

It is with great pleasure that I express my thanks to my colleagues, Mr. S. C. Lo and Mr. Y. H. Wong, for their keen advice and suggestions on enzyme purification techniques. Thanks are also due to Miss P. K. Lee, Miss K. Y. Fung, Miss P. H. Fung, Miss K. C. Ng, Miss Y. L. Shi, Mr. P. C. Leung, Mr. C. M. Chiu, Mr. S. W. To and Mr. K. S. Cheung for their encouragement and kindness during my graduate study.

Special thanks are due to Dr. Vincent E. C. Ooi, supervisor of my Senior Seminar Research Project, for introducing me to scientific research and leading me to the path of graduate study.

Finally, I should thank my beloved parents for their continuous support and care throughout the course of my study.

Table of Contents

Chapter 3 Results

List of Tables

Table

List of Figures

Figure

List of Abbreviations

CBH	cellobiohydrolase
CBO	cellobiose dehydrogenase or cellobiose oxidase
CBQ	cellobiose:quinone oxidoreductase
CMC	carboxymethylcellulose
CMCase	carboxymethylcellulase
DMS medium	Dimethylsuccinate medium
I.U.	International Unit
Native-PAGE	Native Polyacrylamide Gel Electrophoresis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PNP	*p*-nitrophenol
PNPG	*p*-nitrophenyl-ß-D-glucopyranoside
PNPX	*p*-nitrophenyl-ß-D-xylopyranoside
Preparative-PAGE	Preparative Polyacrylamide Gel Electrophoresis
rpm	revolutions per minute
v/v	volume by volume
w/v	weight by volume

CHAPTER 1
INTRODUCTION

1.1 General Background

The recycling of matter is of utmost importance to living systems on earth. According to different estimates, the total annual production of biosynthetic products ranges from 1.0 to 4.0 x 10^{11} tons dry biomass (Tarchevsky and Marchenko, 1991). With respect to carbon, the photosynthetic process is estimated to produce 1.5 x10^{11} tons of dry plant material annually, of which 50% is cellulose (Kubicek *et al.*, 1993). The vast amount of lignocellulosic residues not only provides a means of storing solar energy, but also represents an important energy and material resource for mankind. Over recent decades, scientists have been actively engaged on research into the degradation of this renewable carbon source. Biodegradation, using enzymic hydrolysis instead of chemical hydrolysis, has been studied extensively in recent years due to its advantages over the chemical method.

1.2 Occurrence and Structure of Cellulose

Plant cell walls are complex, heterogeneous structures composed mainly of polymers, such as cellulose, hemicelluloses, and lignins. Cellulose and hemicellulose, respectively, are the two most abundant biopolymers on Earth (Eriksson and Wood, 1985; Dekker, 1985).

Almost half of the biomass synthesized by photosynthetic fixation of carbon dioxide is made up of cellulose. Cotton and wood fibres are the most common sources. Cellulose makes up about 90% of cotton fibres and about 45% of the average wood fibre (Eriksson *et al.*, 1990).

Apart from plants, cellulose is also found in limited groups of fungi, bacteria, invertebrates, and protists (Richmond, 1991).

Cellulose is a linear polymer of up to 14,000 anhydroglucose residues in the chair configuration held together by β-1,4 linkages. Each residue is rotated 180 degrees about the main axis with respect to its neighbouring residues (Figure 1.1) (Coughlan, 1985). Therefore, the basic recurring unit is cellobiose. These cellulose chains, each stabilized by intramolecular hydrogen bonds, are arranged in parallel to form the insoluble microfibril in which the chains are also cross-linked by intermolecular hydrogen bonds. The fibrils aggregate to form bundles and contribute to the great tensile strength of the plant cell walls. Native cellulose fibres consist of highly crystalline regions with well-ordered cellulose chains or fibrils, and amorphous regions with less-ordered chains or fibrils which hence can be degraded more easily by microbial enzymes. The naturally occurring cellulose fibres are often associated with hemicellulose and lignin and the extent of association determines the degradability of the cellulose in lignocellulosic materials since the existence of lignin can be a barrier towards efficient enzymic hydrolysis of cellulose. Karunanandaa *et al.* (1992) conducted experiments to study the degradation of crop residues stover by selected white-rot fungi. Results, however, revealed that the amount of lignin decomposed in crop residues, eg. rice straw and maize, does not generally correlate with changes in digestibility. The presence of silica, which can account for 16% of the dry mass of rice straw, was suggested (Karunanandaa *et al.*, 1992) to have an inhibitory effect on the digestibility of rice straw.

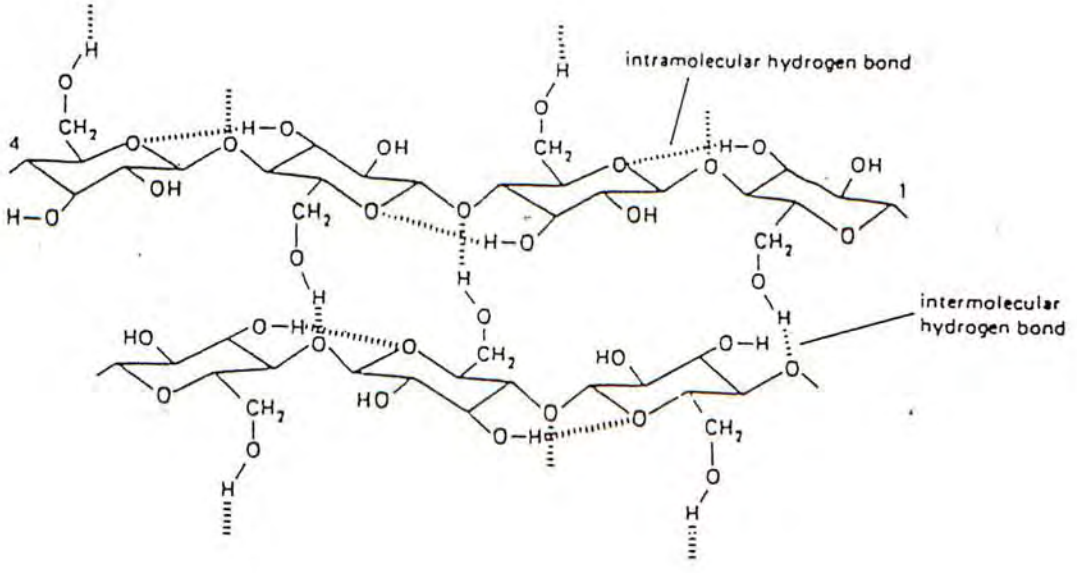

Figure 1.1 Cellulose chains showing the β-1,4-linked residues (Coughlan, 1985).

1.3 Occurrence and Structure of Hemicellulose

Hemicellulose is a collective term ambiguously defined as "those polysaccharides soluble in alkali that are associated with cellulose of the plant cell wall" (Dekker, 1985). The hemicelluloses are composed of both linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. Most hemicelluloses contain two to six of these sugars which may be acetylated or methylated. The predominant types in wood species include the 1,3- and 1,4-ß-D-galactans, 1,4-ß-D-mannans, and 1,4-ß-D-xylans. Softwood and hardwood contains different types of hemicelluloses. The structures of hemicelluloses commonly found in wood species are illustrated in Figure 1.2 (Dekker, 1985).

Hemicelluloses rank next to cellulose in abundance as naturally occurring compounds and account for up to 40% of the total carbohydrate fraction (Gomez De Segura and Fevre, 1993). They are present in both the primary and secondary layers of the plant cell wall. The association between cellulose and hemicelluloses is close, the ribbon-like conformation of both of these molecular types is similar, thus favouring their alignment and packing by means of hydrogen bonds and other non-covalent interactions (Vian and Reis, 1991). Hemicellulose associated with lignin surrounds cellulose fibrils and constitutes a 'barrier', restricting cellulolytic hydrolysis.

1.4 Biodegradation of Cellulose and Hemicelluloses

1.4.1 Cellulolytic and Hemicellulolytic Microorganisms

Degradation of lignocellulosic materials in nature does not normally take place with one organism in monoculture but rather with a consortium of organisms which include both the fungi and bacteria.

An ample number of microorganisms are reported to have the ability to degrade cellulose and hemicelluloses with extracellular and intracellular hydrolytic enzymes. Table 1.1 shows some of the microorganisms used in cellulase and hemicellulases studies.

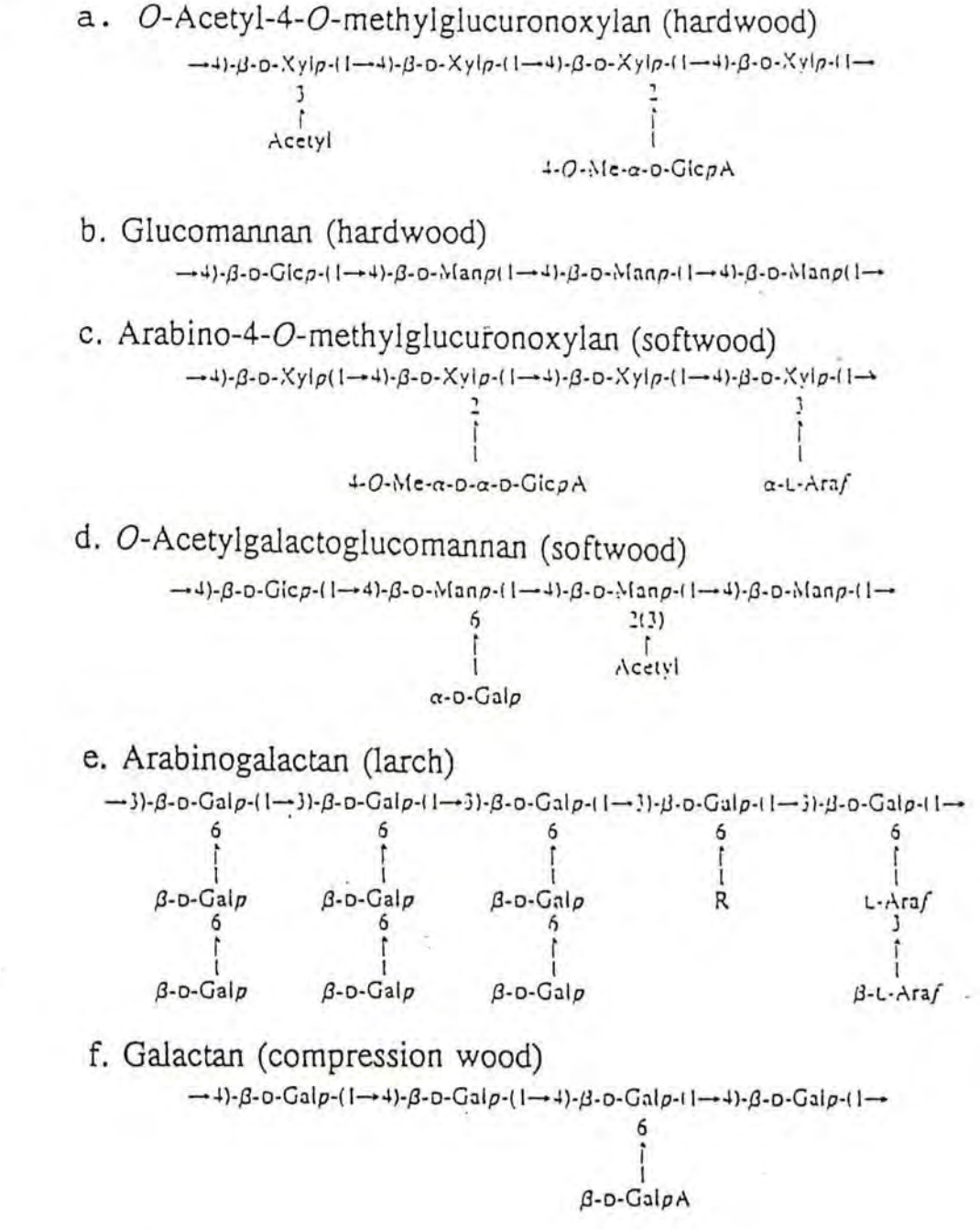

Figure 1.2 Structures of hemicelluloses commonly found in wood species (Dekker, 1985).

Table 1.1 Examples of microorganisms used in cellulase and/or hemicellulases studies.

Species	Literature(s)
Aeromonas caviae	Kubata *et al.* (1993)
Agaricus bisporus	Puls *et al.* (1987) Wood *et al.* (1988)
Amycolata autotrophica	Ball and McCarthy (1989)
Aspergillus awamori	Kormelink and Voragen (1993) Kormelink *et al.* (1993a) Kormelink *et al.* (1993b)
Aspergillus fumigatus	Bailey and Viikari (1993)
Aspergillus niger	Gokhale *et al.* (1992) Kormelink and Voragen (1993) Kormelink *et al.* (1993a) Uchida *et al.* (1992)
Aspergillus niveus	Taj-Aldeen and Alkenany (1993)
Aspergillus oryzae	Bailey and Viikari (1993)
Aureobasidium pullulans	Christov and Prior (1993)
Auricularia polytricha	Lu and Tang (1986)
Bacillus circulans	Ratto *et al.* (1992)
Bacteroides ruminicola	Martin and Akin (1988)
Bacteroides succinogenes	Martin and Akin (1988)
Cellulomonas uda	Dermoun and Belaich (1988)
Clostridium cellulolyticum	Gelhaye *et al.* (1993)
Collybia velutipes	Aschan and Norkrans (1953) Norkrans and Aschan (1953)
Coniophora puteana	Schmidhalter and Canevascini (1992)

Cryptococcus albidus	Biely *et al.* (1980)
Cyathus stercoreus	Karunanandaa *et al.* (1992)
Dichomitus squalens	Karunanandaa *et al.* (1992)
Faenia rectivirgula	Ball and McCarthy (1989)
Fusarium lini	Rao *et al.* (1985)
Lentinula edodes *(Lentinus edodes)*	Leatham (1985) Mishra and Leatham (1990) Moyson and Verachtert (1991) Schmidt and Dittberner (1989)
Micromonospora sp.	Ball and McCarthy (1989)
Neocallimastix frontalis	Gomez De Segura and Fevre (1993)
Neocallimastix sp.	Borneman *et al.* (1989)
Penicillium citrinum	Kuhad and Singh (1993)
Penicillium pinophilum	Wood and McCrae (1986) Wood *et al.* (1989)
Phanerochaete chrysosporium *(Sporotrichum pulverulentum)*	Deshpande *et al.* (1978) Copa-Patino *et al.* (1993) Eriksson and Hamp (1978) Eriksson *et al.* (1993) Kerem *et al.* (1992) Karunanandaa *et al.* (1992) Szakacs Dobozi *et al.* (1992) Uzcategui *et al.* (1991) Westermark and Eriksson (1974)
Piromyces sp.	Borneman *et al.* (1989)
Piromyces sp.	Teunissen *et al.* (1992)
Pleurotus cornucopiae	Tsang *et al.* (1987)
Pleurotus eryngii	Ginterova (1989)
Pleurotus flabellatus	Rajarathnam *et al.* (1979)

Pleurotus ostreatus	Ginterova and Lazarova (1987) Ginterova and Lazarova (1989) Ginterova *et al.* (1992) Kerem *et al.* (1992) Platt *et al.* (1984) Puls *et al.* (1987) Tsang *et al.* (1987)
Pleurotus pulmonarius	Ginterova (1989) Masaphy and Levanon (1992) Moyson and Verachtert (1991)
Pleurotus sajor-caju	Ginterova and Lazarova (1987) Moyson and Verachtert (1991) Royse (1992) Tsang *et al.* (1987)
Pleurotus sapidus	Tsang *et al.* (1987)
Polyporus tulipiferae *(Irpex lacteus)*	Kanda *et al.* (1976a) Kanda *et al.* (1976b) Kanda *et al.* (1976c)
Polyporus versicolor	Westermark and Eriksson (1974)
Saccharomonospora viridis	Ball and McCarthy (1989) McCarthy *et al.* (1985)
Streptomyces flavogriseus	MacKenzie *et al.* (1987)
Streptomyces hygroscopicus	Spear *et al.* (1993)
Streptomyces olivochromogenes	MacKenzie *et al.* (1987)
Streptomyces reticuli	Heupel *et al.* (1993)
Streptomyces sp. A 451	He *et al.* (1993)
Streptomyces sp. no. 3137	Nakanishi *et al.* (1992)
Thermoactinomyces sp.	Hagerdal *et al.* (1979)
Thermomonospora alba	Ball and McCarthy (1989)
Thermomonospora chromogena	Ball and McCarthy (1989) McCarthy *et al.* (1985)

Thermomonospora curvata	Ball and McCarthy (1989) McCarthy *et al.* (1985)
Thermomonospora fusca	Bachmann and McCarthy (1991) Ball and McCarthy (1989) Calza *et al.* (1985) Irwin *et al.* (1993) McCarthy *et al.* (1985) Rothlisberger *et al.* (1992) Walker *et al.* (1992)
Thermomonospora mesophila	Ball and McCarthy (1989)
Thermomyces lanuginosus	Gomes *et al.* (1993)
Trametes gibbosa	Bhattacharjee *et al.* (1992)
Trichoderma koningii	Halliwell and Griffin (1978) Wood and McCrae (1978)
Trichoderma longibrachiatum	Royer *et al.* (1992)
Trichoderma reesei	Bailey *et al.* (1993) Buchert *et al.* (1992) Chahal *et al.* (1992) Chen and Wayman (1992) Fagerstam and Pettersson (1980) Gamerith *et al.* (1992) Irwin *et al.* (1993) Maheswari *et al.* (1993) Nidetzky *et al.* (1993) Nidetzky *et al.* (1994) Poutanen and Puls (1988) Taj-Aldeen (1993) Tilbeurgh *et al.* (1984) Tilbeurgh *et al.* (1989) Uzcategui *et al.* (1991) Walker *et al.* (1992) Woodward *et al.* (1988)
Trichoderma viride	Beldman *et al.* (1985) Beldman *et al.* (1986) Helle *et al.* (1993)
Volvariella volvacea	Buswell *et al.* (1993) Cai *et al.* (1994) Chang and Steinkraus (1982)

The list of organisms in Table 1.1, consists of a variety of bacteria and fungi (including mushrooms). Among them, the most intensively studied fungi are the various species and strains of *Trichoderma* and *Aspergillus*. Edible fungi such as *Lentinula edodes* (Leatham,1985; Schmidt and Dittberner, 1989; Mishra and Leatham, 1990; Moyson and Verachtert, 1991), *Agaricus bisporus* (Puls *et al.*,1987; Wood *et al.*, 1988), *Auricularia polytricha* (Lu and Tang, 1986), *Volvariella volvacea* (Chang and Steinkraus, 1982; Buswell *et al.*, 1993; Cai *et al.*, 1994) and *Pleurotus* sp. (Platt *et al.*, 1984; Puls *et al.*, 1987 and Tsang *et al.*, 1987) are cultured and consumed in huge quantities worldwide and have also been shown to be producers of cellulolytic and/or hemicellulolytic enzymes.

Apart from fungi, bacteria are also members of the cellulolytic and/or hemicellulolytic families. Actinomycetes such as *Thermomonospora* sp. and *Streptomyces* sp. have been widely researched with respect to their production of cellulases and hemicellulases (McCarthy *et al.*,1985; MacKenzie *et al.*, 1987).

1.4.2 Enzymes Involved in Cellulose Degradation

In 1950, Reese *et al.* (1950) first introduced the "C_1-C_X" hypothesis to describe the enzymes of the cellulase system. Accordingly, a so-called C_1 factor carries out a localized loosening of the cellulose chains after which the C_x enzymes hydrolyse the ß-1,4-bonds in those cellulose molecules 'activated' by the hypothetical non-hydrolytic factor (C_1) (Figure 1.3).

Much of the cellulase research was focused for a time on unraveling the nature of the C_1 enzyme. However, there is as yet no evidence to support the existence of such a non-hydrolytic chain-disaggregating enzyme, and this hypothesis must give way to the more

plausible argument that the hydrolysis of native cellulose is the result of the synergistic action of endo- and exo-glucanase enzymes.

cellulose $\xrightarrow[\text{(non-hydrolytic)}]{C_1}$ activated cellulose $\xrightarrow[\text{(hydrolytic)}]{C_x}$ cellobiose $\xrightarrow[\text{(hydrolytic)}]{\beta\text{-glucosidase}}$ glucose

Figure 1.3 Schematic description of the C_1-C_x concept

There is now a consensus of opinion that sequential action of endo- and exo-1,4-ß-glucanase effects the hydrolysis of cellulose. Finally, a 1,4-ß-glucosidase converts water-soluble cellodextrins to glucose. Figure 1.4 shows the simplified diagrammatic mechanism of cellulose degradation.

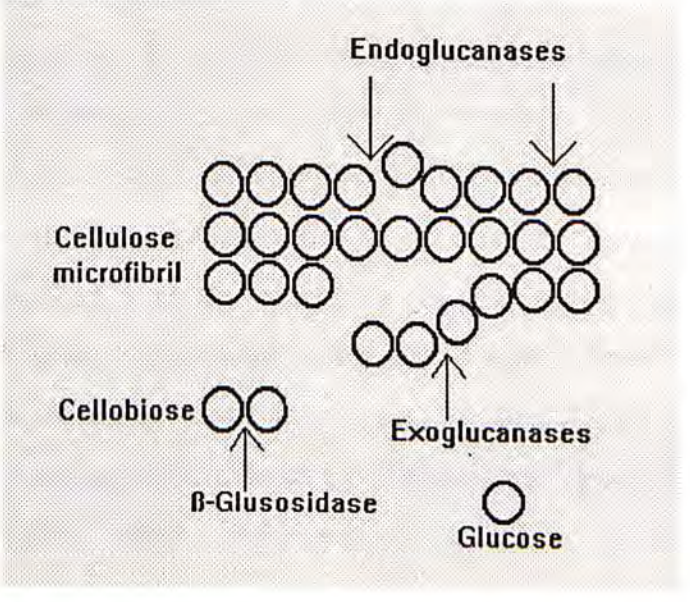

Figure 1.4 Diagrammatic representation of the mechanism of cellulose degradation. The endo-1,4-ß-glucanase attacks cellulose chains at random, splitting ß-1,4 glucosidic linkages. The exo-1,4-ß-glucanase splits off cellobiose/glucose from the non-reducing end of the cellulose. ß-1,4-glucosidase hydrolyzes cellobiose and other soluble cellodextrins to glucose.

1.4.2.1 Endo-1,4-ß-Glucanases

Endoglucanases are defined as enzymes that act randomly on the cellulose chains and open up nonreducing ends for the action of exo-enzymes (Wood and McCrae, 1978; Wood and McCrae, 1986). Substrates of endoglucanases include carboxymethylcellulose (CMC) and phosphoric acid- or alkali-swollen cellulose. It should be noted that neither cotton wool nor crystalline cellulose such as Avicel can be hydrolysed extensively, although exceptions exist. In 1985, Beldman *et al.* (1985) reported a purified endoglucanase (Endo III) from *Trichoderma viride* that showed low activity towards CMC but, of all other purified endoglucanases, this enzyme had the highest specific activity against Avicel. An enzyme with this property is usually classified as an exoglucanase. However, other evidence suggest that this enzyme is clearly an endoglucanase. Firstly, it released cellotriose, cellobiose and glucose from phosphoric acid-swollen cellulose, which indicated its endo-type attack. Second, the enzyme exhibited a synergistic action with exoglucanases. Thirdly, no synergism was observed when the enzyme was working in concert with other endoglucanases.

An endoglucanase with multispecificity was purified by Kanda *et al.* (1976a). They reported that an endoglucanase from *Polyporus tulipiferae* attacked a series of cellooligosaccharides, CMC, as well as insoluble cellulosic substrates such as Avicel. They described this enzyme as an endoglucanase with a less random hydrolytic mechanism and defined it as an endocellulase of 'Avicelase Type'. In 1976, Kanda *et al.* (1976b) also reported a purified endoglucanase of 'carboxymethylcellulase' type that was able to degrade xylan. They further concluded that the xylanase activity of the endocellulase was intrinsic to the cellulase itself. In 1985, Rao *et al.* (1985) also showed that a purified endoglucanase from *Fusarium lini* possessed xylan-degrading ability.

A further example of the broad-substrate specificity of endoglucanase was reported by Wood and McCrae (1978). They found that the endoglucanases of *Trichoderma koningii* could hydrolyse CMC, phosphoric acid-swollen cellulose, cellotetraose and cellopentaose but differed in the rate and mode of attack. Cellobiose, however, was not hydrolysed by any of the endoglucanases, whereas cellotriose is a substrate for all but a low-molecular weight endoglucanase (E1). Glucose, cellobiose and cellotriose are the common products formed after degradation. None of the endoglucanases could solubilize cotton cellulose to any significant extent when acting in isolation, but all could produce changes in the cotton fibre manifested by an increase in the uptake of alkali or a decrease in the tensile strength.

Endoglucanases were shown to be induced by cellobiose at a concentration as low as 1 mg/l and sophorose at a similar concentration (Eriksson and Hamp, 1978). However, the induction time is longer for sophorose than for cellobiose. Components of rice husks (Kuhad and Singh, 1993), cotton-wheat straw (Masaphy and Levanon, 1992), Avicel, oat-spelt xylan and starch-free wheat bran (MacKenzie *et al.*, 1987) were also reported to be inducers of endoglucanases.

In contrast, glucose was shown by many authors as the repressor of endoglucanases. Eriksson and Hamp (1978) reported that glucose concentrations as low as 50mg/ml caused repression of enzyme formation. Mixtures of inducers (cellobiose and sophorose) and repressor (glucose) produced a delayed induction compared with solutions of inducer only. Gokhale *et al.* (1992) proposed that the drastic drop in pH of the medium was responsible for inactivation of cellulase enzymes in the presence of glucose. With urea supplementation, the pH could be maintained and higher endocellulase activity was obtained. Recent studies by Ramos *et al.* (1993) also pointed out that lower rates of hydrolysis resulted from the accumulation of high sugar concentrations. The efficiency of hydrolysis

of cellulosic substrates was enhanced by the removal of soluble sugars liberated during hydrolysis. In the case of the edible mushroom, *Agaricus bisporus*, catabolic repression of endocellulase activity by glucose as well as cellobiose at a concentration of 0.001% was reported (Wood *et al.*, 1988).

Both glycosylated and non-glycosylated endoglucanases have been described (Beldman *et al.*, 1985; Calza *et al.*, 1985) in fungi and bacteria. The molecular weight of the different enzymes range from 23,000 (Rao *et al.*, 1985) to 94,000 (Calza *et al.*, 1985).

1.4.2.2 Exo-1,4-ß-Glucanases

Exoglucanases are defined as enzymes that remove glucose or cellobiose from the non-reducing end of the cellulose chain (Wood and McCrae, 1972; Wood and McCrae, 1978; Wood and McCrae, 1986). Two main exoglucanases are glucanohydrolase and cellobiohydrolase which remove glucose and cellobiose units, respectively. Exoglucanases have no activity against carboxymethylcellulose (CMC) and highly ordered forms of cellulose, but can readily degrade phosphoric acid-swollen cellulose (Wood and McCrae, 1972). Avicel is a common substrate for the detection of exoglucanase (Kanda *et al.*,1976a; Kanda *et al.*, 1976c; Fagerstam and Pettersson, 1980; Wood and McCrae, 1986) and Avicelase is now usually regarded as being synonymous with exoglucanase.

While purified endocellulase from *Polyporus tulipiferae* was shown to have xylanase activity (Kanda *et al.*, 1976b; Rao *et al.*, 1985), purified Avicelase from the same fungus did not show such activity. Immunologically unrelated cellobiohydrolases could be isolated from various fungi such as *Trichoderma reesei* (Fagerstam and Pettersson, 1980) and *Penicillium pinophilum* (Wood *et al.*, 1989; Wood and McCrae, 1986). It was found that some cellobiohydrolases could act

synergistically to degrade microcrystalline cellulose Avicel (Wood and McCrae, 1986; Uzcategui *et al.*, 1991). However, individual enzymes or 'wrong' combinations of enzymes could not render Avicel soluble. It was therefore suggested that the cellobiohydrolases may be two stereospecific enzymes concerned with the hydrolysis of the two different configurations of non-reducing end groups that exist in cellulose (Wood and McCrae, 1986). It was envisaged that the hydrolytic action of one cellobiohydrolase removing cellobiose units successively from one type of non-reducing chain end would expose, on another neighbouring chain, a non-reducing end group with the correct configuration for attack by another stereospecific cellobiohydrolase (Figure 1.5).

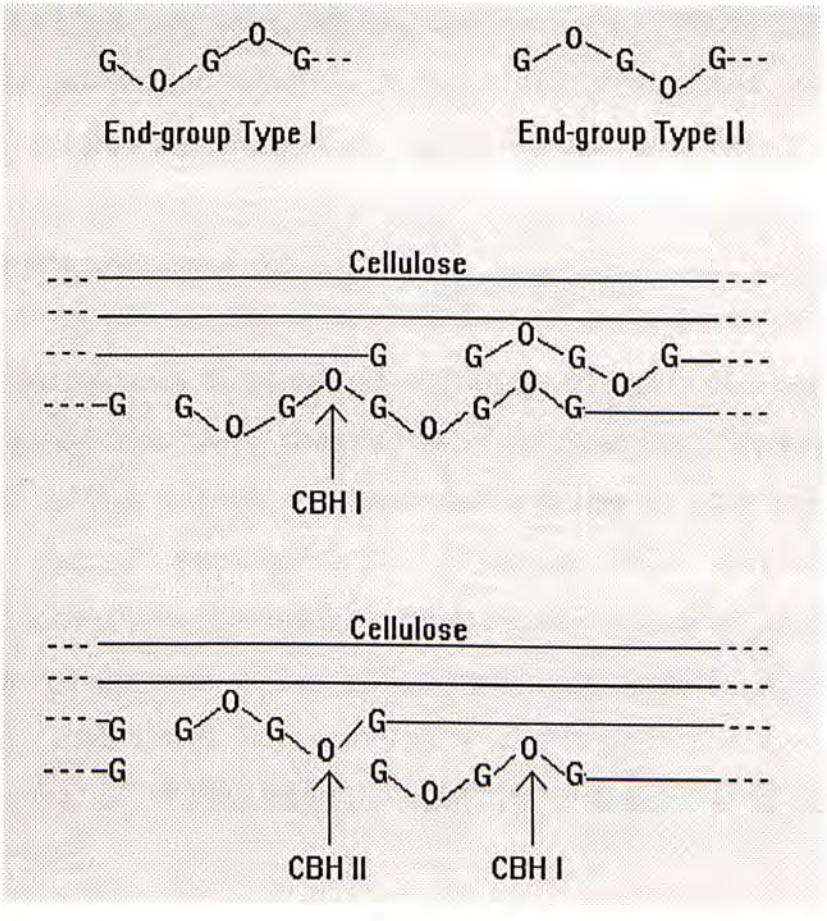

Figure 1.5 Possible mechanism of synergistic action of *P. pinophilum* cellobiohydrolases I (CBHI) and II (CBHII) in solubilizing crystalline cellulose (Wood and McCrae, 1986).

Synergism of this type would not exist during attack on phosphoric acid-swollen cellulose, where most chain ends would be easily accessible to the different cellobiohydrolases (Wood and McCrae, 1986). Assuming such a mechanism for exo-exo synergism, it was further hypothesized that two stereospecific endoglucanases also exist to generate the two sterically different non-reducing end-groups for the cellobiohydrolases to attack.

Cellobiose is an inhibitor of exoglucanases (Eriksson and Wood, 1985). Glucose and cellobiose, being the major product of cellulase action, were also shown to be inhibitors of the synergism between reconstituted mixtures of endo- and exoglucanases (Wood and McCrae, 1978). Exoglucanases so far described are glycoproteins (Wood and McCrae, 1972; Kanda *et al.*, 1976a; Beldman *et al.*, 1985; Uzcategui *et al.*, 1991). Molecular weights of the cellobiohydrolases range from 45,000 to 62,000 (Beldman *et al.*, 1985; Eriksson and Wood, 1985).

1.4.2.3 ß-Glucosidases

ß-Glucosidases catalyze the hydrolysis of cellobiose and aryl and alkyl-ß-D-glucosides with the liberation of glucose. Studies on the production of this enzyme are important because of its involvement in the final step of saccharification. Common assay methods for ß-glucosidases include the measurement of the release of glucose from cellobiose (Deshpande *et al.*, 1978; Helle *et al.*, 1993), or the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl-ß-D-glucopyranoside (PNPG) (Deshpande *et al.*, 1978; Hagerdal *et al.*,1979; Heupel *et al.*, 1993; Taj-Aldeen, 1993).

Both extracellular (Deshpande *et al.*, 1978; Platt *et al.*, 1984; Bhattacharjee *et al.*, 1992; Chen and Wayman, 1992; Masaphy and Levanon, 1992; Kuhad and Singh, 1993; Spear *et al.*, 1993; Taj-Aldeen, 1993) and cell associated (Hagerdal *et al.*, 1979; Heupel *et al.*,

1993; Maheswari *et al.*, 1993; Spear *et al.*, 1993) ß-glucosidases have been reported. However, Spear *et al.* (1993) found that although significant amounts of intracellular ß-glucosidase activity could be detected in *Streptomyces hygroscopicus*, the amount of extracellular ß-glucosidase activity was extremely low. Hagerdal *et al.* (1979) also reported that little ß-glucosidase activity could be found in culture filtrates of *Thermoactinomyces* spp., while the culture solids contained the major part of the activity of the whole culture. Since the major end product resulting from the action of extracellular cellulases on cellulose is cellobiose, the low extracellular ß-glucosidase activity but higher cell associated (intracellular or cell-wall-bound) enzyme activity suggests that the mechanism for the assimilation of cellulose may involve the initial extracellular conversion to cellobiose followed by a cell associated conversion of cellobiose to glucose (Spear *et al.*, 1993).

ß-Glucosidases are induced by cellobiose (Heupel *et al.*, 1993) or cellulose (Deshpande *et al.*, 1978). Interestingly, with cellobiose as the sole carbon source, only cell wall-bound enzymes were produced. For extracellular secretion, cellulose seems to be a necessary carbon source. Production of ß-glucosidase can also be induced by using Avicel as a carbon source (Heupel *et al.*, 1993). Cotton-wheat straw (Masaphy and Levanon, 1992), starch (Chen and Wayman, 1992; Taj-Aldeen, 1993), carboxymethylcellulose (CMC) (Bhattacharjee *et al.*, 1992) and rice husks (Kuhad and Singh, 1993) in the culture medium can also induce ß-glucosidases. The presence of ß-glucosidase also increased the activity of synergistic mixtures of endo- and exo-glucanases due to the removal of end-product inhibition (Irwin *et al.*, 1993).

Inhibitors of ß-glucosidases include glucose (Bhattacharjee *et al.*, 1992; Heupel *et al.*, 1993), glucono-lactone and histidine (Heupel *et al.*, 1993). Both glycosylated (Wood, 1991) and non-glycosylated ß-glucosidases (Heupel *et al.*, 1993) have been isolated. The molecular

weights range from 39,800 (Eriksson and Wood, 1985) to 182,000 (Deshpande *et al.*, 1978).

1.4.2.4 Oxidative Enzymes

Apart from hydrolytic enzymes, oxidative enzymes also participate in cellulose degradation. For example, the white-rot fungus, *Phanerochaete chrysosporium* produced cellobiose:quinone oxidoreductase (CBQ) (Westermark and Eriksson, 1974), also called cellobiose dehydrogenase, and cellobiose oxidase (CBO) (Ayers *et al.*, 1978) when grown on cellulose. The presence of these oxidative enzymes in wood-degrading white-rot fungi but not in brown-rot fungi indicates possible roles in lignin degradation. Brown-rot fungi are defined as those that grow on wood and cause rapid and extensive depolymerization of the cellulose and hemicellulose components and a limited, but definite, modification of its lignin (Eriksson *et al.*,1990), such as the removal of part of the methoxy groups. Most brown-rot fungi are unable to grow on pure cellulose. However, a few species of the family Coniophoraceae are able to thrive in culture with cellulose as sole carbon source. In 1992, Schmidhalter and Canevascini (1992) reported the presence of cellobiose dehydrogenase in culture filtrates of *Coniophora puteana,* a brown-rot fungus, grown on amorphous cellulose. This at least suggested that the enzymic mechanism used by brown-rot fungi to bring about wood (lignin) degradation is in some instances, similar to that of ligninolytic white-rot fungi. Whatever their role in lignin degradation, the two different cellobiose oxidoreductases were postulated by Eriksson *et al.* (1993) to have some physiological role in cellulose degradation:

(1) oxidation of cellulose, which introduces carboxyl groups resulting in a disordering of the crystalline structure of cellulose due to disruption of hydrogen bonds between cellulose chains;

(2) conversion of cellobiose, which inhibits cellulose hydrolyzing enzymes, into cellobionic acid;

(3) oxidation of reducing end groups in cellulose to prevent reformation of glycosidic bonds broken by cellulases;

(4) direct energy uptake by coupling with electron transfer chains on the cell walls, generate active species to disrupt the crystalline structure of cellulose, thus facilitating hydrolysis by cellulases.

1.4.3 Synergistic Action between Cellulolytic Enzymes

Synergism between the enzymes involved in cellulose degradation has been known for some time. It is now well-established that degradation of native hydrogen-bond-ordered cellulose is dependent to a large extent on the synergistic action of endoglucanases, exoglucanases and ß-glucosidases. Synergism in the degradation of crystalline cellulose between endo- and exo-glucanases has been well documented by many authors (Halliwell and Griffin, 1978; Wood and McCrae, 1978; Wood and McCrae, 1986; Beldman *et al.*, 1985; Beldman *et al.*, 1986; Woodward *et al.*, 1988; Wood *et al.*, 1989; Walker *et al.*, 1992; Irwin *et al.*, 1993; Nidetzky *et al.*, 1993; Bailey *et al.*, 1993).

Based on their experimental results on enzyme synergism, Wood and McCrae (1972) suggested that the process of solubilization is initiated by the endoglucanases, and that the new chain ends are subsequently attacked by cellobiohydrolase. With the discovery that only definite kinds of exoglucanases can act synergistically with the endoglucanases to degrade cotton fibre, Wood and McCrae (1978) suggested that the endoglucanases have to form an enzyme-enzyme complex with the 'right' exoglucanases on the surface of the cellulose chains to prevent reformation of glucosidic linkage after the attack by endoglucanases.

Another type of synergism, exo-exo synergism, that is a synergism between two exoglucanases has been demonstrated by Fagerstam and Pettersson (1980) during the degradation of Avicel. Wood and McCrae (1986), Uzcategui *et al.* (1991), and Nidetzky *et al.* (1994) also provided evidence for exo-exo synergism in the degradation of Avicel. Wood and McCrae (1986) suggested that the cellobiohydrolases may be two stereospecific enzymes concerned with the hydrolysis of the two different configurations of non-reducing end groups existing in cellulose.

Synergism between endoglucanases (endo-endo synergism) was also reported, but the substrates used for degradation studies were restricted to carboxymethylcellulose (Rao *et al.*, 1985).

Synergistic action is not restricted to enzymes belonging to the same species. Wood and McCrae (1986) reported a cross-synergism between the exoglucanase of *Trichoderma koningii* and the exoglucanase of *Fusarium solani* in solubilizing Avicel. The exoglucanase of *T. koningii* also acted synergistically to solubilize crystalline cellulose with the endoglucanase of *F. solani*. Synergism between fungal and bacterial cellulases was also described by Walker *et al.* (1992). They found that the exoglucanase of *Thermomonospora fusca* and the endoglucanase of *Trichoderma reesei* could effect the fragmentation and the production of reducing sugars from Avicel.

Woodward *et al.* (1988) showed that synergism between cellulase components appears to be independent of their ratio in a reaction mixture and dependent only on their individual concentrations. They suggested that at high concentrations of cellulase, co-operation between cellulase components will be hindered because all the binding sites for which the components appear to compete will be saturated. However, experimental evidence obtained by Nidetzky *et al.* (1993) revealed that the synergistic action between the cellulases decreased when the substrate concentration was increased in the case of non-

saturating enzyme concentration. This result seems to contradict the findings of Woodward *et al.* (1988). Nidetzky *et al.* (1993) envisaged that synergism depends strongly on the type of cellulose used and is low on easily accessible cellulose. They speculated that the cellulases mainly attack those parts of the substrate that are more easily accessible, especially when, at high substrate concentrations, the number of these sites is non-limiting. The preferential interaction of the cellulases with substrate sites easily available for hydrolysis could explain the decrease in synergistic action observed with increasing substrate concentrations.

Due to the potentials of cellulases in various industrial applications, many researches have been conducted to enhance the cellulase productivity by microorganisms. Some of the work included the identification of new species, isolation of hyperproducing mutants of existing strains and cloning of the appropriate genes. Considerable success has been achieved in enhancing enzyme productivity especially with *Trichoderma reesei* mutants (Morawetz *et al.*, 1992). Moreover, a cosmid gene library was constructed in *Escherichia coli* from genomic DNA isolated from the ruminal anaerobe *Fibrobacter succinogenes* AR1. Clones were screened on carboxymethylcellulose, and colonies that produced clearing zones were identified (Cavicchioli and Watson, 1991). The complete nucleotide sequence of the cellulase-encoding gene *cel*H of *Clostridium thermocellum* was determined (Yague *et al.*, 1990). In addition, Wang *et al.* (1993) reported the cloning of the gene coding for the *Clostridium thermocellum* cellulase S_2 into *E. coli* and the nucleotide sequence and sequence analysis of the respective DNA region.

Some of the other molecular biological work which has been done on the cellulolytic enzymes are described in the review articles by Coughlan (1985) and Kubicek *et al.* (1993).

1.4.4 Enzymes Involved in Hemicellulose Degradation

Hemicellulases specifically attack glycans that make up the backbone chain of the hemicelluloses. Since the predominant types of polysaccharides that make up the hemicelluloses include the 1,3- and 1,4-ß-D-galactans, 1,4-ß-D-mannans and 1,4-ß-D-xylans, typical hemicellulases are therefore ß-D-galactanases, ß-D-mannanases, and ß-D-xylanases. Exoglycosidases such as α- and ß-D-galactosidases, ß-D-mannosidases, α-L-arabinosidases, and ß-D-xylosidases are also capable of hydrolyzing short-chain or monosaccharide appendages associated with the main hemicellulosic backbone chain (Dekker, 1985).

1.4.4.1 Endo-1,4-ß-Xylanases

Xylan is the major component of the hemicellulose of plant cell walls and constitutes up to 35% of the total dry weight of higher plants (Kubata *et al.*, 1993). Therefore, enzymes involved in the degradation of native xylan, namely, endoxylanases, have received much attention and are the most extensively studied hemicellulases. Endoxylanases attack the linear polyxylose chain of xylan by hydrolyzing the 1,4-ß-D-xylopyranosyl linkages (Dekker, 1985).

Common substrates for the assay of endo-1,4-ß-xylanases include oat spelt xylan (McCarthy *et al.*, 1985; Teunissen *et al.*, 1992; Copa-Patino *et al.*, 1993; He *et al.*, 1993; Kubata *et al.*, 1993), beechwood xylan (Ratto *et al.*, 1992; He *et al.*, 1993) and larchwood xylan (Borneman *et al.*, 1989; Royer *et al.*, 1992; Srivastava and Srivastava, 1994).

Xylobiose (Kubata *et al.*, 1993), xylotriose and xylotetraose (He *et al.*, 1993) were shown to be products of xylan degradation by the enzymes. Usually, xylan was degraded to a mixture of oligomers (McCarthy *et al.*, 1985). Gomez De Segura and Fevre (1993) isolated and purified two ß-endoxylanases produced by *Neocallimastix frontalis.*

Both enzymes exhibited carboxymethylcellulase activity, and one was adsorbed on crystalline cellulose. In 1992, Gamerith *et al.* (1992) used hemicellulosic wastes with low cellulose content as the growth substrate for the production of cellulase-poor xylanases by *Trichoderma reesei* RUT C-30. However, Royer *et al.* (1992) provided evidence that the cellulase activity of purified xylanase on carboxymethylcellulose (CMC) was due to the contamination of the assay substrate with xylan.

Endoxylanases are inducible enzymes. Production of endoxylanase can be induced during growth on substrates such as lactose, starch, cellobiose and filter paper cellulose (Teunissen *et al.,* 1992). Beech xylan (Ratto *et al.*, 1992; Rothlisberger *et al.*, 1992), oat spelt xylan (McCarthy *et al.*, 1985; Rothlisberger *et al.*, 1992), xylose and glucose (Srivastava and Srivastava, 1994), and corn stalks (Szakacs Dobozi *et al.,* 1992) are also inducers for endoxylanases. Other complex substrates such as bagasse, wheat bran and wheat straw are good inducers for xylanases (MacKenzie *et al.*, 1987; Teunissen *et al.,* 1992). Bailey *et al.* (1993) observed the best production of xylanase on insoluble, unsubstituted beech xylan rather than the soluble glucuronoxylan and concluded that poor substrate availability leads to effective enzyme induction. In addition, cultivation conditions such as unusually high pH also resulted in poor substrate availability and therefore led to enzyme induction.

Xylobiose was shown to be a metabolisable inducer and is effective at low concentration and constant availability to cells. At high concentrations, the inductive effect of xylobiose is less pronounced because of catabolic repression by degradation products. Methyl ß-D-xylopyranoside was also found to serve as a non-utilizable inducer of ß-xylanase (Biely *et al.,* 1980). Xylanase production on highly purified microcrystalline cellulose (Avicel) by *Phanerochaete chrysosporium* suggested that at least part of the xylanase enzyme system is constitutive (Szakacs Dobozi *et al.*, 1992).

Xylanases are also subject to repression by various substances. Endoxylanase activity was inhibited by xylobiose, but not by cellobiose or monomeric sugars (Ball and McCarthy, 1989). Phenolic monomers such as *p*-coumaric acid and ferulic acid were shown to inhibit *Bacteroides succinogenes* xylanase (Martin and Akin, 1988).

1.4.4.2 ß-Xylosidases

Another group of well-studied hemicellulolytic enzymes are the ß-xylosidases. These enzymes hydrolyze xylooligosaccharides to xylose and are essential for the complete degradation of xylans (Eriksson *et al.*, 1990).

ß-Xylosidase activity is determined by measuring the *p*-nitrophenol (PNP) released from *p*-nitrophenyl ß-D-xylopyranoside (PNPX) (Poutanen and Puls, 1988; Copa-Patino *et al.*, 1993) or xylose released from xylobiose (Szakacs Dobozi *et al.*, 1992).

Production of ß-xylosidase can be induced by growth substrates such as rice bran, oat spelt, wheat flour, larchwood, birchwood (Kormelink and Voragen, 1993), beech xylan (Ratto *et al.*, 1992) and corn stalk (Szakacs Dobozi *et al.*, 1992). The presence of ß-xylosidase was found to enhance endoxylanase activities probably by relieving end product inhibition on endoxylanase (Bachmann and McCarthy, 1991). On the other hand, ß-xylosidase was competitively inhibited by xylose which is another example of product inhibition (Poutanen and Puls, 1988; Uchida *et al.*, 1992).

In 1988, Poutanen and Puls (1988), purified a ß-xylosidase from *Trichoderma reesei* which also showed α-arabinofuranosidase activity.

1.4.4.3 Other Xylanolytic Enzymes

Since xylan is a branched heteropolymer and highly substituted with L-arabinose/L-arabinofuranose, methylglucurono- and acetyl groups, other enzymes have to act in concert for the complete degradation of xylan.

It has been shown that the presence of substituent groups such as arabinofuranosyl groups on the xylan backbone clearly inhibit the action of endoxylanases on glycosidic linkages in the vicinity of the site of substitution (Kormelink and Voragen, 1993). It was suggested that the presence of these substituent groups prevents a good fit between the substrate and the enzyme binding site.

Side branch-cleaving enzyme such as α-glucuronidase was found to be produced by edible mushrooms such as *Agaricus bisporus* and *Pleurotus ostreatus* (Puls *et al.*, 1987). 1,4-ß-D-Arabinoxylan arabinofuranohydrolase and acetyl xylan esterase are also reported (Ratto *et al.*, 1992; Szakacs Dobozi *et al.*, 1992; Uchida *et al.*, 1992; Kormelink and Voragen, 1993).

1.4.5 Synergistic Action between Hemicellulolytic Enzymes

Since hemicelluloses are highly branched and substituted polysaccharides, synergism might be expected to exist between the different hemicellulases in the degradation process. Indeed, synergistic actions have been studied and reported. Poutanen and Puls (1988) reported synergistic activities between xylanase and esterase from *Streptomyces olivochromogenes* and the ß-xylosidase from *Trichoderma reesei* in the degradation of birchwood hemicellulose. Copa-Patino *et al.* (1993) also reported a synergism between xylanase and ß-xylosidase from *Phanerochaete chrysosporium* in the degradation of oat spelt xylan. Cooperative activity of endoxylanase, α-arabinofuranosidase, ß-xylosidase and acetyl esterase was evidenced by Bachmann and McCarthy (1991) during the degradation of oat spelt xylan and straw,

and by Kormelink and Voragen (1993) on rice bran, oat spelts, wheat flour, larchwood, and birchwood. In the case of α-glucuronidase, Puls *et al.* (1987) showed that this enzyme acted synergistically with xylanases to liberate glucuronic acid from glucuronic acid substituted xylooligomers. Kormelink and Voragen (1993) suggested that the presence of large amounts of substituents may hinder the formation of enzyme-substrate complexes and thus impede enzymic hydrolysis. Enzymes able to release these substituents, in particular arabinofuranosyl substituents, xylopyranosyl and galactopyranosyl substituents, and acetyl groups, are therefore essential for the complete degradation of heteroxylans.

1.5 *Flammulina velutipes*

Flammulina velutipes, also called the 'winter mushroom' due to its appearance on natural substrates during early autumn and late winter months, was first cultivated in China around A.D. 800-900 (Chang and Miles, 1989). Because of its unique taste, texture and pleasing appearance, cultivation of the mushroom has undergone development. In 1986, it was estimated that production of the mushroom amounted to 100,000 tons (Chang, 1991) which ranked sixth in the worldwide production of edible mushrooms (Chang and Miles, 1989). In 1989, production had increased to over 200,000 tons. Japan was formerly the major *Flammulina*-growing country, but now cultivation of the mushroom has spread to Taiwan, Korea and China. In 1989, Japan produced up to 83,200 tons of *F. velutipes* with an average wholesale price of US$4.48 per kilogramme (Chang, 1991).

Flammulina velutipes was originally cultivated on natural wood logs but the quality of harvested fruitbodies was usually inferior. Now, the fungus is usually cultivated on sawdust and rice bran mixtures

which has been recognized as an ideal method for commercial exploitation (Wood and Smith, 1987).

In spite of its economic importance, little is known about the ability of *Flammulina velutipes* to degrade the cellulose, hemicellulose and lignin components of the growth substrates. Detailed investigations on lignocellulolytic enzymes produced by edible basidiomycetous fungi are restricted to only a few species, such as, *Lentinula edodes, Pleurotus* spp. and *Agaricus bisporus.* Information on the production of lignocellulolytic enzymes such as cellulases and hemicellulases by *F. velutipes* and its ability to utilize the major polymeric components of a particular substrate as a source of nutrition for growth and fruiting is unfortunately limited.

1.6 Aims of the Present Investigation

(1) to determine the optimal conditions for the assay of cellulolytic and xylanolytic enzymes activities of *Flammulina velutipes* by growing the fungus in submerged culture using purified components of lignocellulose as the major carbon sources.

(2) to investigate the cellulolytic and xylanolytic enzyme production profiles of the fungus by assaying individual enzymic activities during growth over timecourses.

(3) to determine enzyme induction patterns by growing the fungus in media containing potential enzyme inducers.

(4) to partially purify the different cellulase species produced by the fungus grown on selected inducer(s) in (3).

CHAPTER 2

MATERIALS AND METHODS

2.1 Organism

Flammulina velutipes (Fl-5) was obtained from the Department of Biology Collection at The Chinese University of Hong Kong (C.U.H.K.) and maintained on Potato Dextrose Agar (PDA) (Difco) at 4°C with periodic subculture.

2.2 Culture Medium

The basal culture medium (DMS medium) contained (g/l):

KH_2PO_4	0.2g
$MgSO_4 \cdot 7H_2O$	0.05g
$CaCl_2 \cdot 2H_2O$	0.013g
2,2-Dimethylsuccinate (DMS)	1.46g
L-Asparagine	1.0g
NH_4NO_3	0.5g
Trace elements (Kirk *et al.*, 1978)	1ml
Vitamins (Kirk *et al.*, 1978)	0.5ml

The pH was adjusted to 4.8 with 1 M *o*-phosphoric acid or 1 M KOH and the medium was sterilized by Millipore filtration.

Avicel (Sigmacell Type 20), 1% w/v, or birchwood xylan (Sigma), 1% w/v, served as carbon source.

The trace elements and vitamin solution were prepared according to Kirk *et al.* (1978). They contained the following per litre of distilled water:

Trace elements		Vitamin solution	
nitrilotriacetate	1.5g	biotin	2mg
$MgSO_4 \cdot 7H_2O$	3.0g	folic acid	2mg
$MnSO_4 \cdot H_2O$	0.5g	thiamine·HCl	5mg
NaCl	1.0g	riboflavin	5mg
$FeSO_4 \cdot 7H_2O$	100mg	pyridoxine·HCl	10mg
$CoSO_4$	100mg	cyanocobalamine	0.1mg
$CaCl_2$	82mg	nicotinic acid	5mg
$ZnSO_4$	100mg	DL-calcium pantothenate	5mg
$CuSO_4 \cdot 5H_2O$	10mg	*p*-aminobenzoic acid	5mg
$AlK(SO4)_2$	10mg	thioctic acid	5mg
H_3BO_3	10mg		
$NaMoO_4$	10mg		

2.3 Determination of the Optimal Growth pH of *Flammulina velutipes*

Mycelial discs were cut with a cork borer (7mm diameter) from the actively growing margin of a 2-week old culture of *F. velutipes* growing on PDA and inoculated into 150-ml Erlenmeyer flasks each containing 30 ml of filter-sterilized culture medium adjusted to different pH values. Triplicate cultures were prepared for each pH value. The culture medium for this experiment contained the following per litre of citrate-phosphate buffer (5mM citric acid and 10mM K_2HPO_4) adjusted to the pH values 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.4, 6.8, 7.2 and 7.6:

Glucose	10.0g
$MgSO_4 \cdot 7H_2O$	0.05g
$CaCl_2 \cdot 2H_2O$	0.013g
L-Asparagine (Sigma)	1.0g
NH_4NO_3	0.5g
Yeast extract (Difco)	5.0g
Trace elements (Kirk *et al.*, 1978)	1.0ml
Vitamins (Kirk *et al.*, 1978)	0.5ml

Cultures were incubated at room temperature under static conditions and harvested after 2 weeks. Final pH values of the cultures were recorded. Harvested mycelia were washed with distilled water and dried at 105°C overnight. Mycelial dry weights were recorded.

2.4 Preparation of Inoculum, Cultivation and Harvest of Fungal Cultures

For experiments to determine the optimal conditions for cellulolytic and xylanolytic enzyme activity, stock cultures were inoculated into 50 ml of Potato Dextrose Broth (PDB) (Difco), pH 4.8, in 150-ml Erlenmeyer flasks. After 3 weeks of static incubation at room temperature, the cultures were transferred to a sterile blender and homogenized in 50 ml distilled water for two 15 second periods. A 5 ml aliquot was transferred to 125ml DMS medium containing 1% (w/v) Avicel (Sigmacell Type 20) or 1% (w/v) birchwood xylan (Sigma) in a 500-ml Erlenmeyer flask.

After 20 days incubation in an orbital incubator shaker at 25°C, 150 rpm, the cultures were harvested by centrifugation (Beckman J2-M1 Centrifuge) at a speed of 27,000×g for 15 minutes. Culture supernatants and mycelial pellets were retained for the enzymic investigations.

For time-course experiments, 1 ml of the mycelial homogenate prepared as described above was inoculated into 40 ml Avicel- or birchwood xylan-supplemented (1% w/v) DMS medium contained in 150 ml Erlenmeyer flasks. Triplicate cultures were harvested at intervals and the activity of cellulolytic and xylanolytic enzymes determined.

2.5 Enzyme Assays

2.5.1 Exo-1,4-ß-glucanase

Exoglucanase (Avicelase) activity was assayed by measuring the reducing sugar released from the hydrolysis of Avicel (Sigmacell Type 20) using the Somogyi-Nelson reagent (Wood and Bhat, 1988; Somogyi, 1951). Table 2.1 shows the composition of the Somogyi-Nelson reagent.

Table 2.1 Somogyi-Nelson Reagent

Somogyi Reagent (per L dH_2O)		Nelson Reagent (per L dH_2O)	
Na_2SO_4	180g	Ammonium molybdate	50g
K/Na Tartrate	12g	Na_2H arsenate	6g
Na_2CO_3	24g	Conc. H_2SO_4	42ml
$NaHCO_3$	16g		
$CuSO_4 \cdot 5H_2O$	4g		

The Somogyi reagent was stored at room temperature, while the Nelson reagent was stored at 37°C for 2 days in a brown glass bottle and then at room temperature.

The reaction mixture consisted of 0.8 ml 1% (w/v) Avicel, 1.7 ml 50mM phosphate buffer (KH_2PO_4-KOH buffer) or citrate-phosphate buffer (0.1M citric acid, 0.2M Na_2HPO_4) and 0.5 ml culture supernatant. The assay mixture was maintained at 40°C for 15 minutes (enzyme activity was shown to be linear with time over this period - see p.56) at which time the reaction was stopped by adding 1 ml of Somogyi reagent. The mixture was boiled for 15 minutes and then cooled to ambient. Nelson reagent (1 ml) was added and, after mixing on a vortex, the resultant mixture was centrifuged at 3,400×g for 15 minutes (Sorvall Superspeed Centrifuge). The absorbance of the supernatant was read at 520nm (Milton Roy Spectrometer 601) and the amount of reducing sugar released determined from a standard curve prepared using D-glucose. One unit of enzyme activity was defined as the amount of enzyme releasing 1μmol of reducing sugar (glucose equivalents) per minute under the assay conditions. Figure 2.1 shows the standard curve of glucose for the measurement of reducing sugar by Somogyi-Nelson reagent.

2.5.2 Endo-1,4-ß-glucanase

As mentioned in Section 1.4.2.2, exoglucanase normally has no activity against carboxymethylcellulose (although enzyme multispecificity may occur). Therefore, endoglucanase (Carboxymethylcellulase or CMCase) activity was determined by measuring the reducing sugar released from the hydrolysis of carboxymethylcellulose (CMC) (Sigma, medium viscosity) using the Somogyi-Nelson method. The procedure was the same as that used for assaying exoglucanase except that Avicel was replaced by 1% (w/v) CMC and the reaction mixtures were maintained at 50°C for 15 minutes. One unit of enzyme activity was defined as the amount of enzyme releasing 1μmol of reducing sugar (glucose equivalents) per minute under the assay conditions.

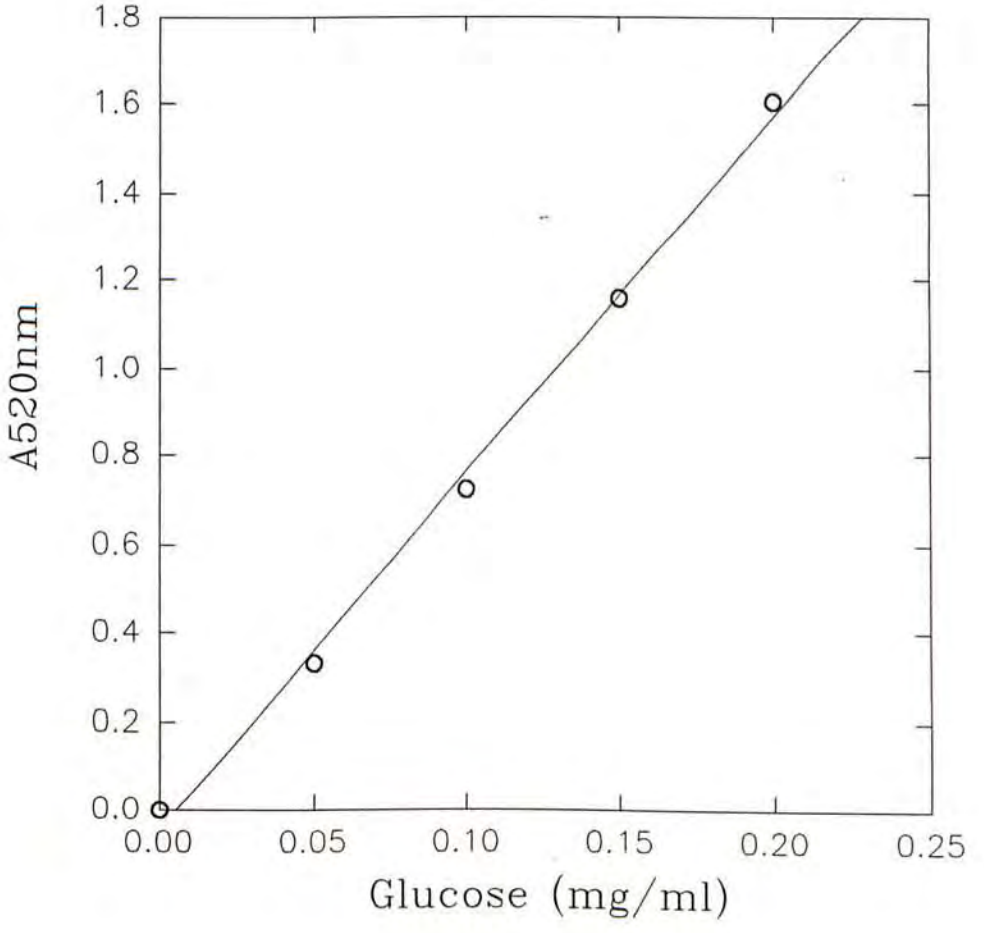

Figure 2.1 Standard Curve of Glucose for the Measurement of Reducing Sugar by Somogyi-Nelson Reagent.

2.5.3 Endo-1,4-ß-Xylanase

Endoxylanase activity was determined by measuring the reducing sugar released from the hydrolysis of birchwood xylan (Sigma) using the Somogyi-Nelson method. The procedure was the same as that for exoglucanase except that Avicel was replaced by 1% (w/v) birchwood xylan and reaction mixtures were incubated at 40°C for 20 minutes. The amount of reducing sugar released was determined from a standard curve prepared using D-xylose. One unit of enzyme activity was defined as the amount of enzyme releasing 1μmol of reducing sugar (xylose equivalents) per minute under the assay conditions. Figure 2.2 shows the standard curve of xylose for the measurement of reducing sugar by the Somogyi-Nelson method.

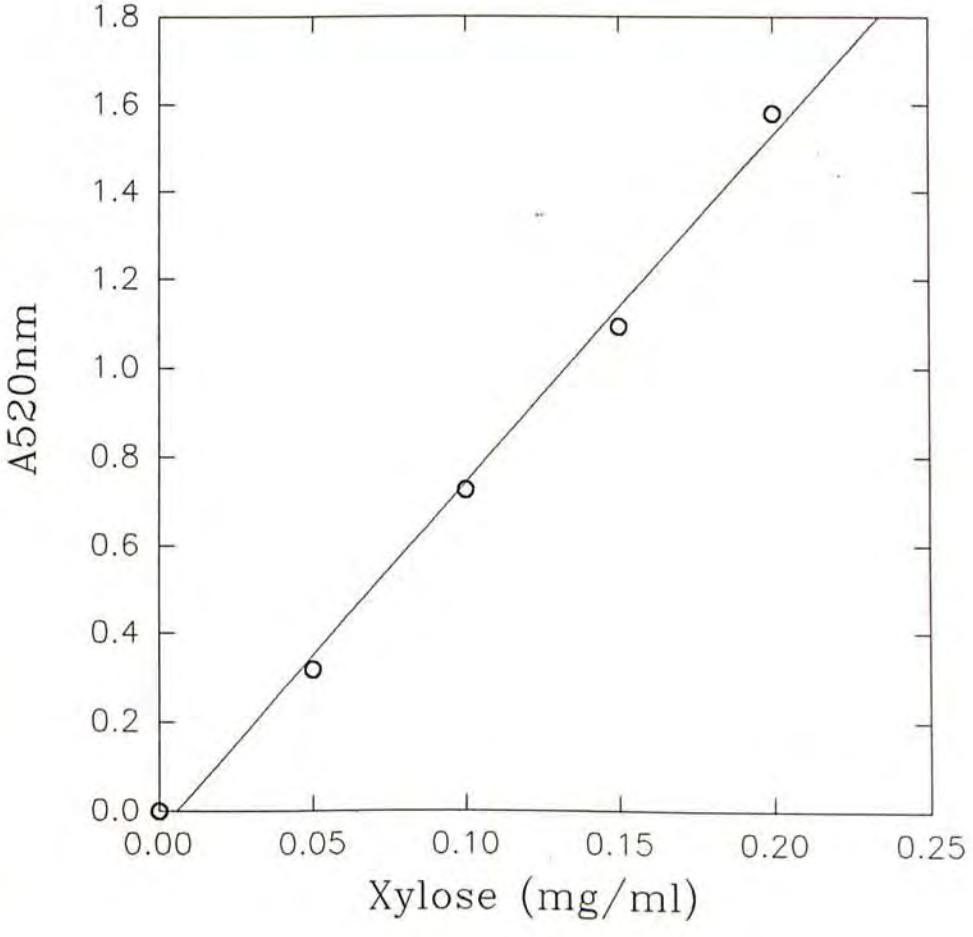

Figure 2.2 Standard Curve of Xylose for the Measurement of Reducing sugar by Somogyi-Nelson Reagent.

2.5.4 Extracellular ß-Glucosidase

ß-Glucosidase activity was determined by measuring the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl-ß-D-glucopyranoside (PNPG) (Sigma).

The reaction mixture consisted of 50 µl 40 mM PNPG, 0.9 ml citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) and 50 µl culture supernatant. After incubation at 50°C for 15 minutes, the reaction was stopped by adding 3 ml of 1 M Na_2CO_3. The yellow colour formed was read at 400nm and the *p*-nitrophenol released was determined from a standard curve prepared using PNP (Sigma). One unit of enzyme activity was defined as the amount of enzyme releasing 1µmol of PNP per minute under the assay conditions. Figure 2.3 shows the standard curve of PNP for the measurement of ß-glucosidase activity.

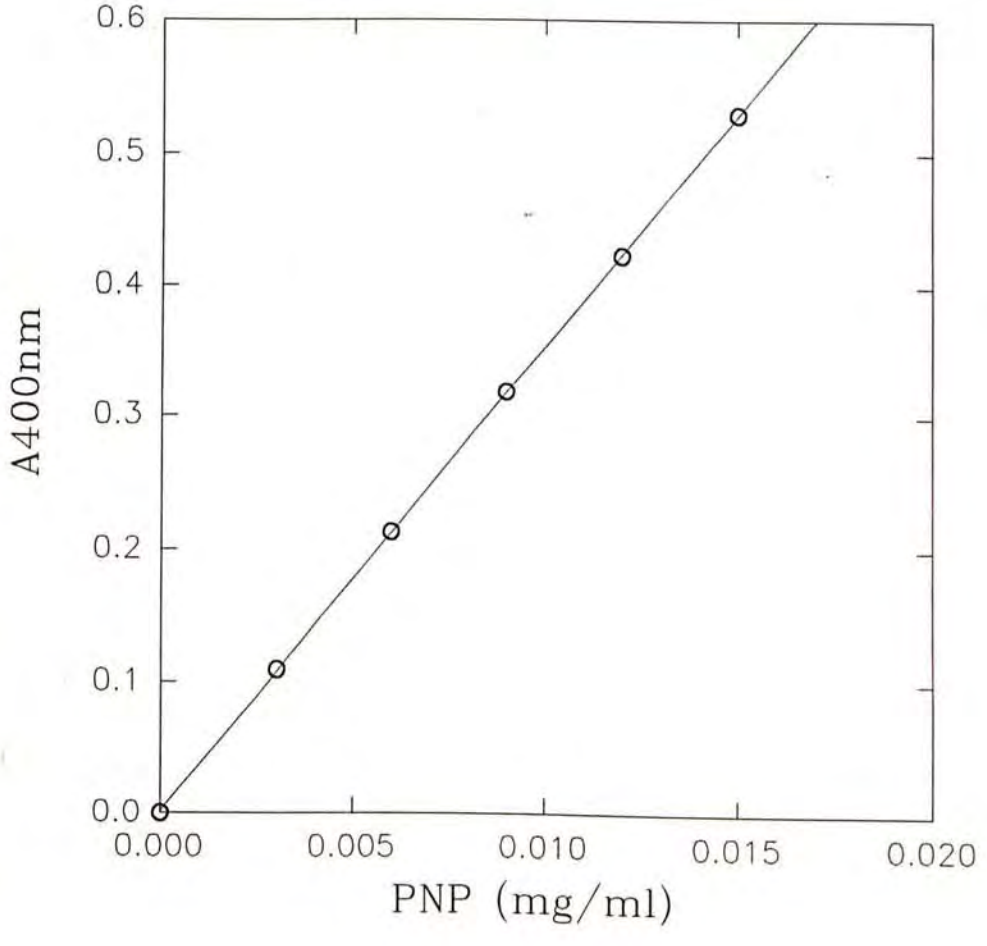

Figure 2.3 Standard Curve of PNP for the Measurement of ß-Glucosidase Activity

2.5.5 Cell-Associated ß-Glucosidase

The procedure for the assay of cell-associated ß-glucosidase was the same as that for extracellular ß-glucosidase except that the 50 µl culture supernatant was replaced by 50 µl enzyme extract. The enzyme extract was prepared as follows: Harvested mycelial pellets were washed with distilled water to remove any extracellular enzymes and then homogenized in 4 ml citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) by a hand homogenizer. The homogenate was then centrifuged (MSE Microcentrifuge) at 11,600×g for 10 minutes at 4°C. The supernatant (enzyme extract) obtained was used for the assay of cell-associated ß-glucosidase activity. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of PNP per minute under the assay conditions.

2.5.6 Extracellular ß-Xylosidase

ß-Xylosidase activity was determined by measuring the release of PNP from *p*-nitrophenyl-ß-D-xylopyranoside (PNPX) (Sigma).

The procedure for the assay of extracellular ß-xylosidase was the same as that for extracellular ß-glucosidase except that PNPG was replaced by 50 µl 25 mM PNPX. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol PNP per minute under the assay conditions.

2.5.7 Cell-Associated ß-Xylosidase

The preparation of enzyme extract and the procedure for the assay of cell-associated ß-xylosidase were the same as those for the cell-associated ß-glucosidase except that PNPG was replaced by 50 µl 25 mM PNPX. One unit of enzyme activity was defined as the amount

of enzyme releasing 1 μmol of PNP per minute under the assay conditions.

2.6 Determination of Optimal Temperatures for Cellulolytic and Xylanolytic Enzymes

Culture supernatants or enzyme extracts were incubated in 50 mM phosphate buffer (KH_2PO_4-KOH buffer), pH 6 (for Avicelase, CMCase and xylanase) or citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6 (for extracellular and cell-associated β-glucosidase and β-xylosidase) with the appropriate substrate at 20, 30, 40, 50, 60, 70 or 80°C for 30 minutes. The amount of product released were determined according to the enzyme assays described.

In order to confirm that enzyme activity was linear with time over the duration of enzyme assay, assays were terminated at various time intervals and the amount of product released plotted against time.

2.7 Determination of the Optimal pH for Enzyme Reaction

Culture supernatants or enzyme extracts were incubated with the appropriate substrate in citrate phosphate buffer adjusted to different pH values. Reactions were conducted at the optimal assay temperatures as determined in Section 2.6 and the time during which enzymic reaction is linear with time. The amounts of products released were determined.

2.8 Protein Determination

Protein content was determined by the method of Lowry *et al.* (1951) using the reagents shown in Table 2.2.

To determine protein in culture supernatants or enzyme extracts, 2 ml Reagent C was mixed with 0.4 ml sample. After 10 minutes at room temperature, 0.4 ml reagent D was added and mixed within 1-2 seconds. After a further 30 minutes, the absorbance was read at 690nm and the amount of protein determined from a standard curve prepared using bovine serum albumin (Fraction V, Sigma). Figure 2.4 shows the standard curve of bovine serum albumin for the determination of protein content using the method by Lowry *et al.*.

Table 2.2 Reagents Prepared for Protein Determination

Reagent A: Na_2CO_3 (2%) and NaOH (0.4%)
Reagent B: $CuSO_4 \cdot 5H_2O$ (1%) and NaK-tartrate (2%)
Reagent C: Reagent A: Reagent B (50:1, v/v) (Mix just prior to use)
Reagent D: Folin-Ciocalteu's phenol reagent (Merck) : distilled water (1:1, v/v) (Freshly prepared)

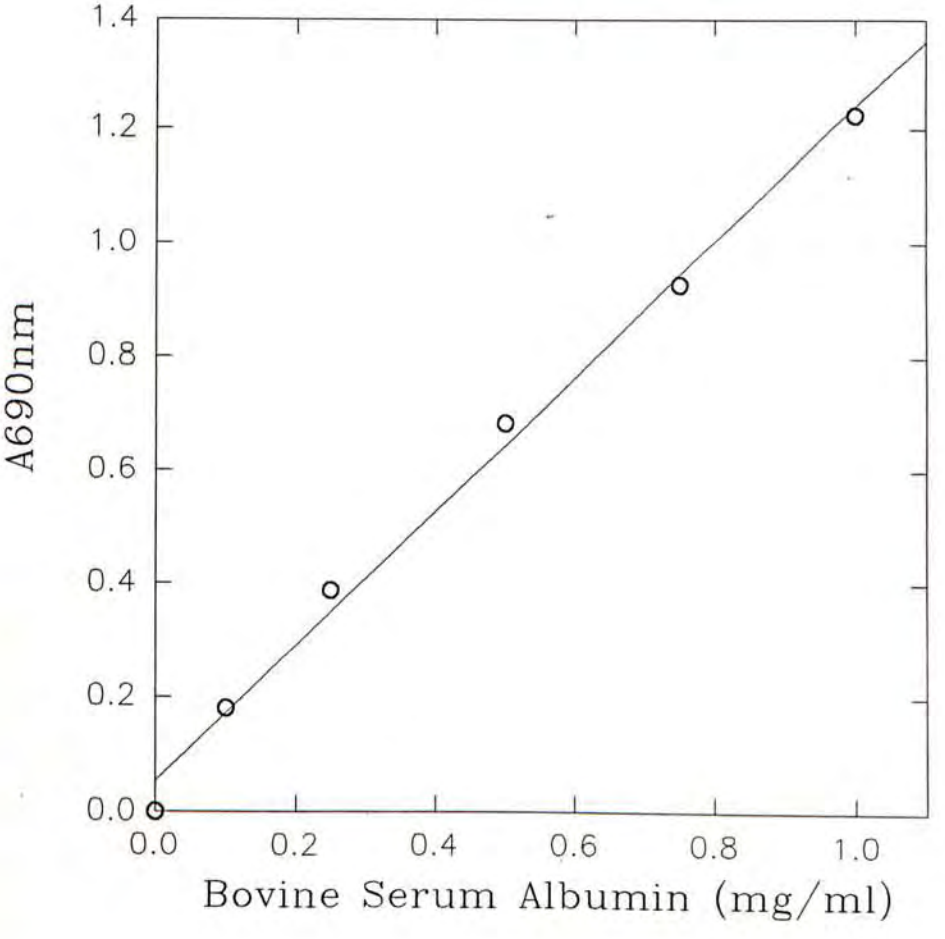

Figure 2.4 Standard Curve of Bovine Serum Albumin for the Determination of Protein Content.

2.9 Determination of Enzyme Induction Patterns

Aliquots (20 ml) of mycelial homogenate (prepared as described in 2.4) were transferred to two 1-L flasks containing 300 ml PDB, pH4.8, supplemented with 0.5% yeast extract. Cultures were incubated at 25°C for 17 days in an orbital shaker operated at 150 rpm. Mycelial pellets (about 3mm in diameter) were harvested by centrifugation at 3,400×g for 5 minutes, washed three times with sterile distilled water and transferred aseptically to the induction media.

Induction medium consisted of 400 ml DMS (pH 4.8) supplemented with 1% (w/v) substrate in a 1-L flask. Substrates examined were Avicel, CMC, filter paper (Whatman No.1), cotton wool (absorbent cotton wool, Snow Mountain, China), rice straw and birchwood xylan. Triplicate flasks were prepared using each substrate. Filter paper, cotton wool and rice straw were treated as follows before use: filter paper was cut into small discs of 0.6 mm in diameter using a paper punch. Cotton wool was cut into pieces approximately 1 cm by 1 cm in size. Rice straw was cut into fragments about 1 cm in length, blended in a blender, and then sieved. The size of the sieved fragments used in the culture medium was less than 0.9 mm in diameter. Inoculated flasks were incubated at 25°C, in an orbital shaker (Gallenkamp) operated at 150rpm. Samples were taken at intervals, centrifuged at 3,400×g (Sorvall Superspeed Centrifuge) for 10 minutes to remove mycelial pellets and insoluble substrates, and assayed for enzyme activity. Supernatants from cultures containing Avicel, CMC, filter paper, cotton wool or rice straw were assayed for exoglucanase, endoglucanase and ß-glucosidase activities. Supernatants from cultures containing rice straw or birchwood xylan were assayed for xylanase and ß-xylosidase activities.

2.10 Elucidation of Cellulase Production Patterns in *F. velutipes*

Eleven 2-L flasks each containing 600 ml DMS medium supplemented with 1% (w/v) Avicel were inoculated as in Section 2.9. Exoglucanase, endoglucanase and ß-glucosidase activities were monitored periodically and flask contents harvested after 18 days when enzyme levels were close to maximum.

Flasks contents were filtered through cheese cloth to remove the mycelial pellets and the filtrate centrifuged at 27,000×g (Beckman J2-M1 Centrifuge) for 15 minutes to remove suspended particles. The supernatant obtained was then concentrated by ultrafiltration (Pellicon membrane, molecular weight cut-off 10,000 daltons) and lyophilized. The resulting dry powder was stored at 4°C until further use.

2.10.1 Native Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis (Native-PAGE) (0.75mm, 10%) was performed using the Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). 0.014g of the dry powder in Section 2.10 was dissolved in 1 ml distilled water and mixed at 4:1 (v/v) with loading buffer containing 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose before loading into the wells. Electrophoresis was carried out at constant voltage (200V) and at 4°C for about 30 minutes in Tris-Glycine buffer (0.025 M Tris, 0.2 M Glycine), pH 8.3.

2.10.2 Activity Staining for Endoglucanases

After electrophoresis, the gel was overlaid with 1.5% (w/v) agarose (Sigma) containing 0.5% (w/v) CMC in citrate-phosphate buffer (0.1M citric acid, 0.2M Na_2HPO_4), pH7, and incubated at 50°C. After 1 hour, the agarose overlay was separated from the gel and stained for

30 minutes in 1% (w/v) Congo red. NaCl (1 M) was then used to destain the overlay for another 30 minutes with frequent changes of NaCl. Colourless regions on the agarose overlay corresponded to the location of endoglucanases in the gel.

2.10.3 Activity Staining for ß-Glucosidases

After electrophoresis, the gel was soaked in citrate phosphate buffer (0.1M citric acid, 0.2M Na_2HPO_4), pH 5.4 for 5 minutes. 1ml 40mM PNPG was then added and spread over the surface of the gel which was then incubated at 50°C for 30 minutes. $NaCO_3$ (1 M) was added to stop the reaction. Yellow bands represented the location of ß-glucosidases in the gel.

2.10.4 Protein Staining

Separated proteins in the slab gels after electrophoresis were silver-stained using the Silver Stain Kit (Bio-Rad).

2.10.5 Preparative Polyacrylamide Gel Electrophoresis

Preparative polyacrylamide gel electrophoresis (Preparative-PAGE) (1mm, 10%) was performed using the PROTEAN II Electrophoresis Cell (Bio-Rad). 0.014g of the dry powder in Section 2.10 was dissolved in 1ml distilled water and mixed at 4:1 (v/v) with loading buffer containing 0.25% bromophenol blue and 40% sucrose before loading into the wells. Electrophoresis was carried out at constant current (24mA for each slab gel) and at 4°C for about 4 hours in Tris-Glycine buffer (0.025 M Tris, 0.2 M Glycine), pH 8.3.

2.10.6 Separation of Proteins and Partial Purification of Different Cellulase Species after Preparative Polyacrylamide Gel Electrophoresis

A vertical strip was cut out from the gel after preparative-PAGE and silver-stained for proteins. Horizontal strips of the gel corresponding to the protein bands were then cut out and individual strips placed in dialysis tubing containing Tris-Glycine buffer (0.025 M Tris, 0.2 M Glycine), pH8.3. To elute the proteins from the gel, the tubing was placed into a MINNIE Submarine Agarose Gel Unit (Hoefer Scientific Instruments) containing Tris-Glycine buffer (0.025 M Tris, 0.2 M Glycine), pH8.3, and a voltage of 200V applied for 1 hour. The contents of the tubing were then dialysed against water overnight. Samples after dialysis were assayed for exoglucanase, endoglucanase and extracellular ß-glucosidase activities. A 0.75mm 10% Native-PAGE was also performed using the samples obtained after dialysis to confirm the number of protein band(s) cut out from the preparative gel.

CHAPTER 3

RESULTS

3.1 Determination of the Optimal pH for Fungal Growth

Figure 3.1 shows the effect of pH on the growth of *Flammulina velutipes* in buffered medium. As shown in the figure, maximum growth was recorded at pH 4.8. At more acidic pH values, a marked decline in mycelial growth was observed. Similarly, at pH values higher than 4.8, mycelial growth initially dropped very sharply but then remained relatively constant over the pH range 5.6 to 7.6. Thus, in all subsequent experiments, the pH of culture media was adjusted to 4.8.

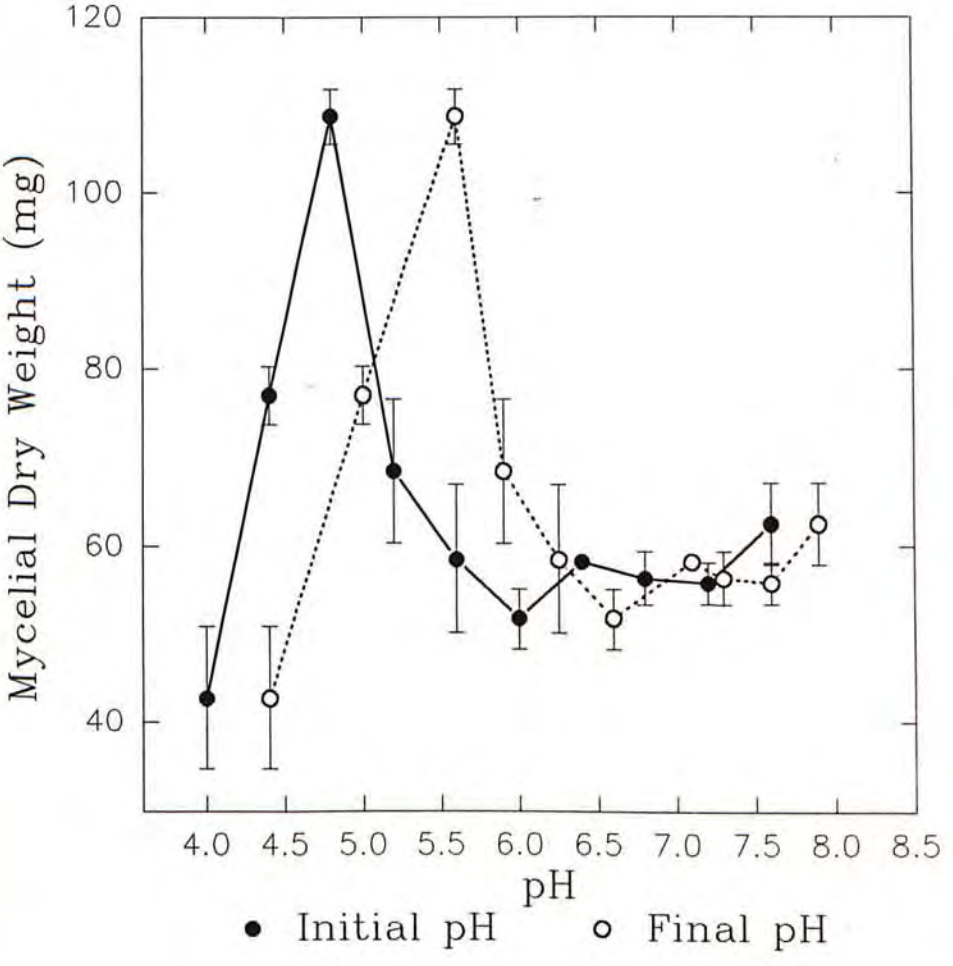

Figure 3.1 Effect of pH on the Growth of *Flammulina velutipes*. 7 mm-diameter mycelial discs of a fungal culture grown on PDA were inoculated into filter-sterilized culture medium adjusted to different pH values (initial pH values). After 2 weeks of incubation at room temperature under static conditions, the cultures were harvested. The final pH values of the medium and the mycelial dry weights were recorded. Values shown represent the mean of triplicate cultures and the error bars are the standard errors.

3.2 Determination of the Optimal Temperature for Cellulolytic and Xylanolytic Enzyme Activity

The effect of temperature over the range 20-80°C on the activity of exoglucanase, endoglucanase, xylanase, extracellular and cell-associated ß-glucosidase and ß-xylosidase from *F. velutipes* is shown in Figures 3.2 to 3.8.

The optimal temperature for the activity of exoglucanase (Figure 3.2), endoglucanase (Figure 3.3), extracellular ß-glucosidase (Figure 3.4) and cell-associated ß-glucosidase (Figure 3.5) were determined to be 40°C, 50°C, 50°C, and 50°C, respectively. The xylanolytic enzymes, ß-xylanase (Figure 3.6), extracellular ß-xylosidase (Figure 3.7) and cell-associated ß-xylosidase (Figure 3.8) exhibited highest activities at 40°C, 60°C and 60°C, respectively. It should be noted that the exoglucanase was relatively heat-stable since 50-60% of the activity observed at 40°C was retained at 60-80°C. In contrast, endoglucanase, and extracellular and cell-associated ß-glucosidases, lost more than 90% of their activity at 70-80°C. Both extracellular and cell-associated ß-glucosidases exhibited very similar temperature-activity profile.

Xylanase from *F. velutipes* was also relatively heat-stable. Over 50% of enzyme activity observed at 40°C was retained at 70-80°C. On the other hand, an abrupt drop in the activities of the extracellular and cell-associated ß-xylosidases was observed above 60°C. For this reason, both xylosidases were routinely assayed at 50°C since 60°C was too close to the denaturation temperature of the enzymes. For all other enzymes, the temperature-optimum determined in these experiments was selected as the assay temperatures in subsequent studies.

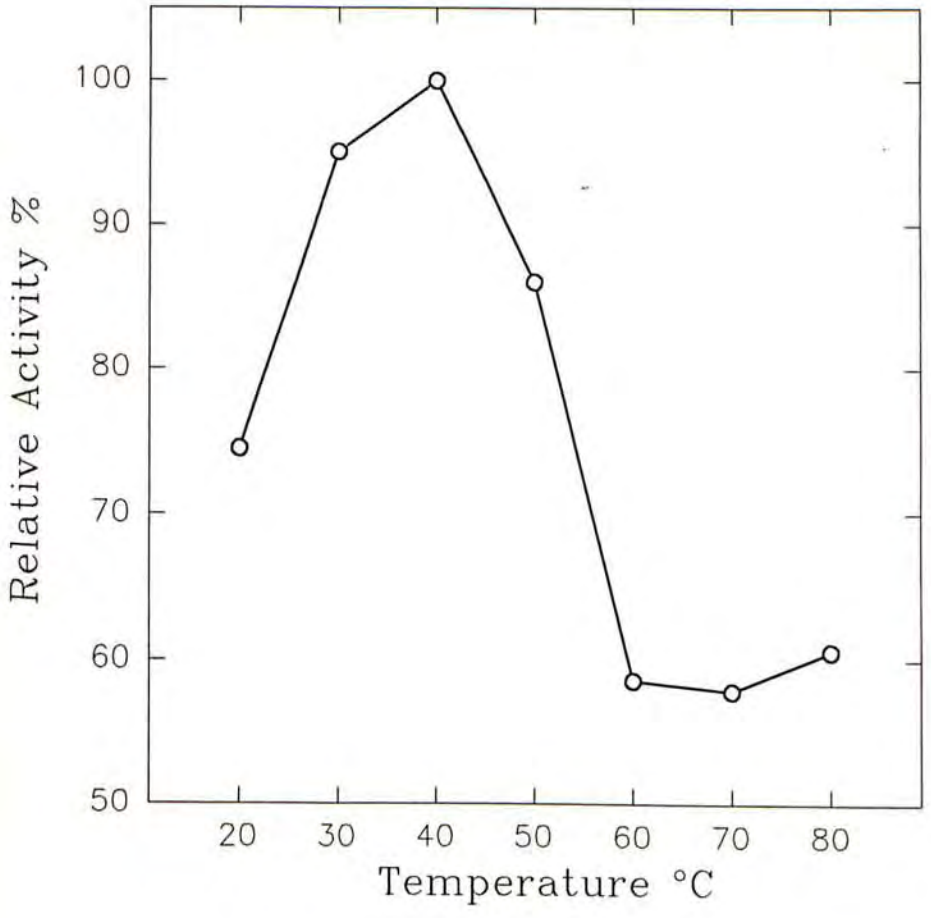

Figure 3.2 Effect of Temperature on the Activity of Exoglucanase. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with Avicel at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 40°C.

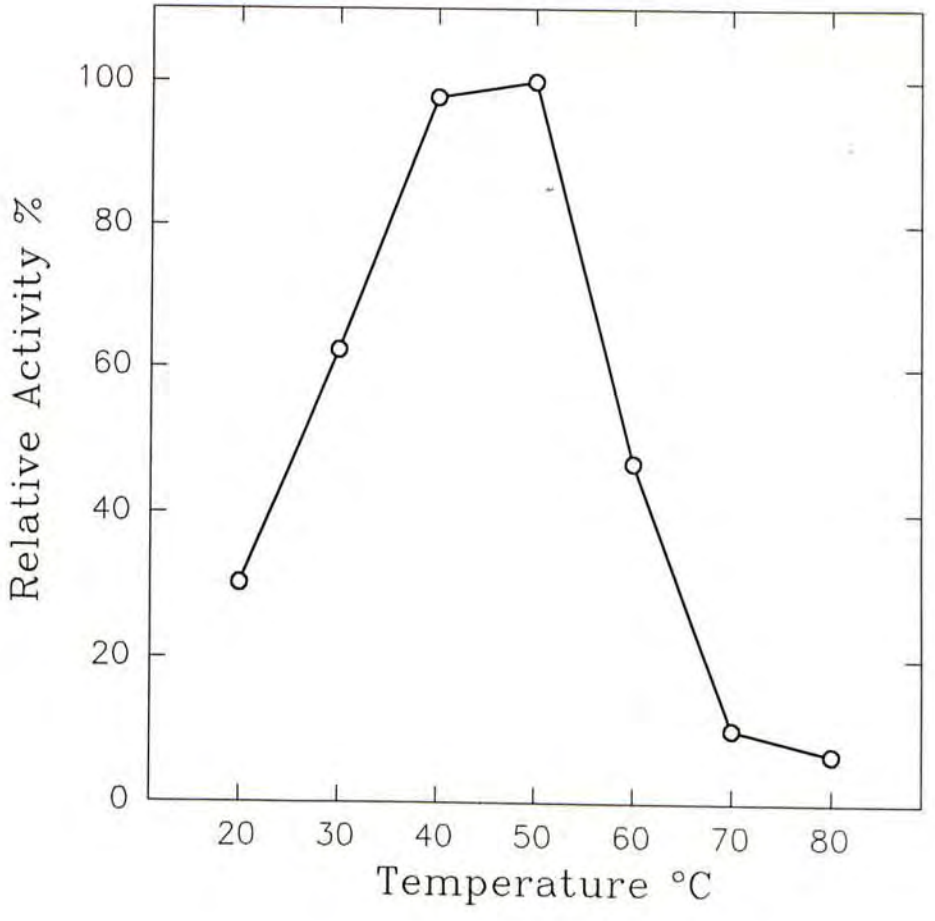

Figure 3.3 Effect of Temperature on the Activity of Endoglucanase. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with CMC at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 50°C.

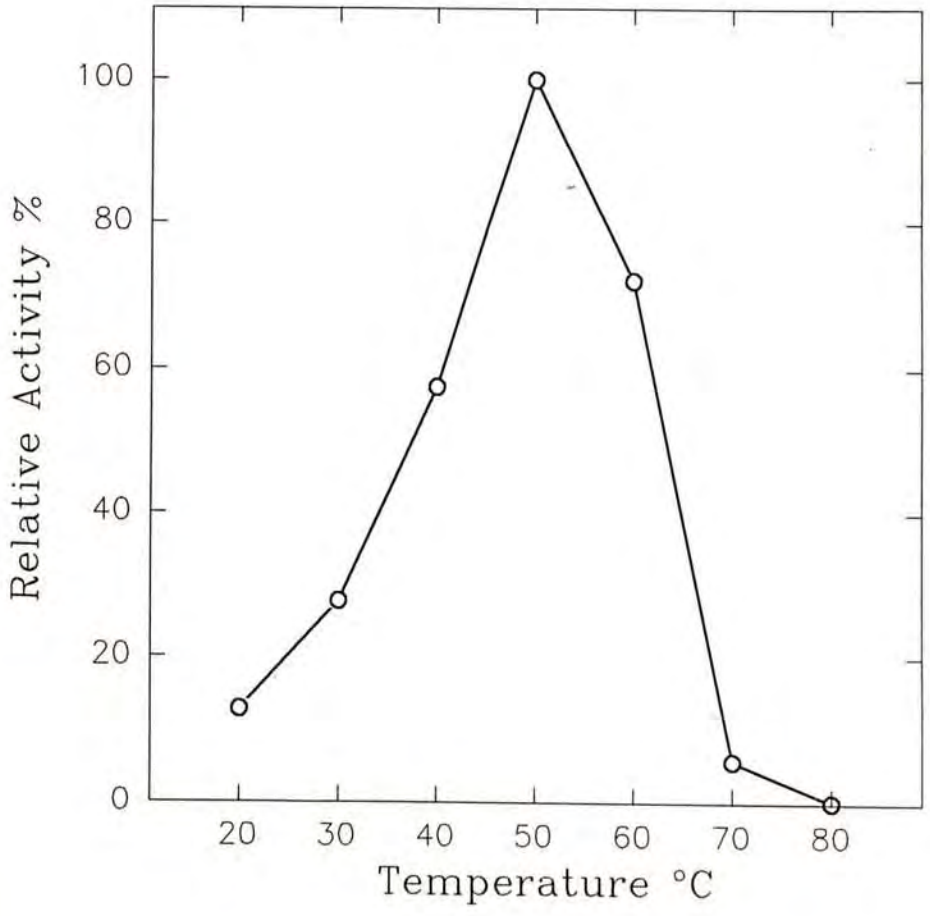

Figure 3.4 Effect of Temperature on the Activity of Extracellular ß-Glucosidase. Culture supernatants were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPG at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 50°C.

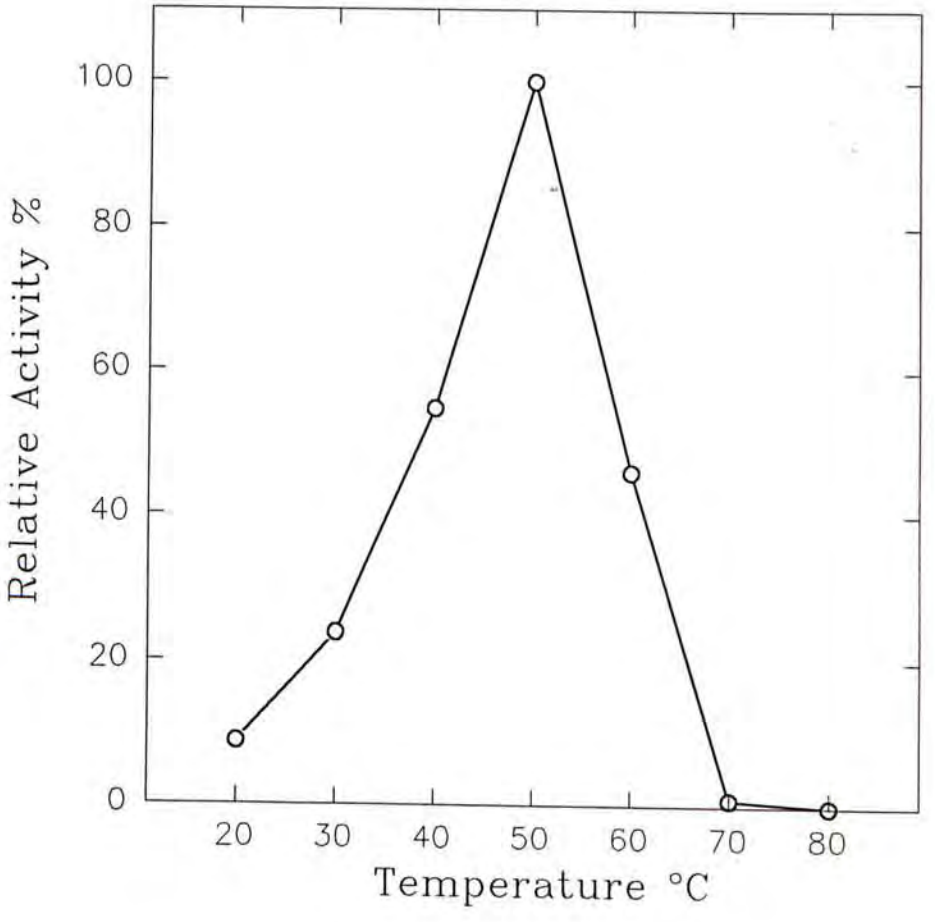

Figure 3.5 Effect of Temperature on the Activity of Cell-associated ß-Glucosidase. Enzyme extracts were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPG at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 50°C.

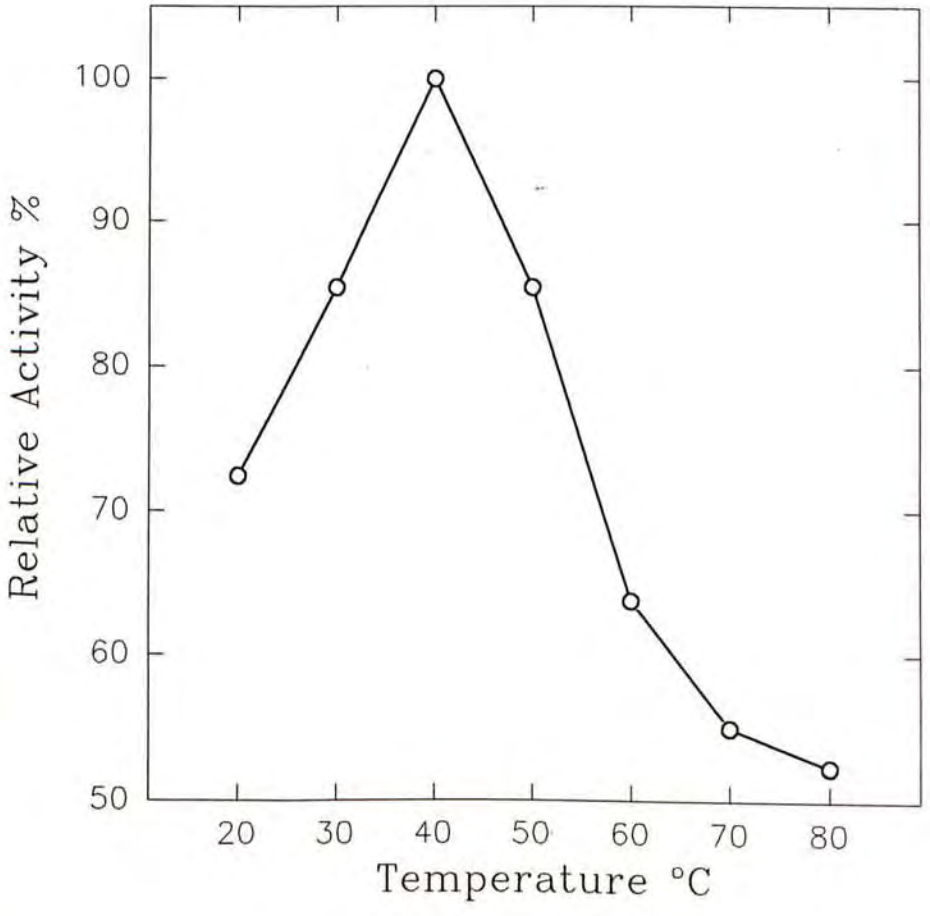

Figure 3.6 Effect of Temperature on the Activity of ß-Xylanase. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with birchwood xylan at various temperatures for 30 miuntes. The amount of product released were determined. Highest activity (100%) was observed at 40°C.

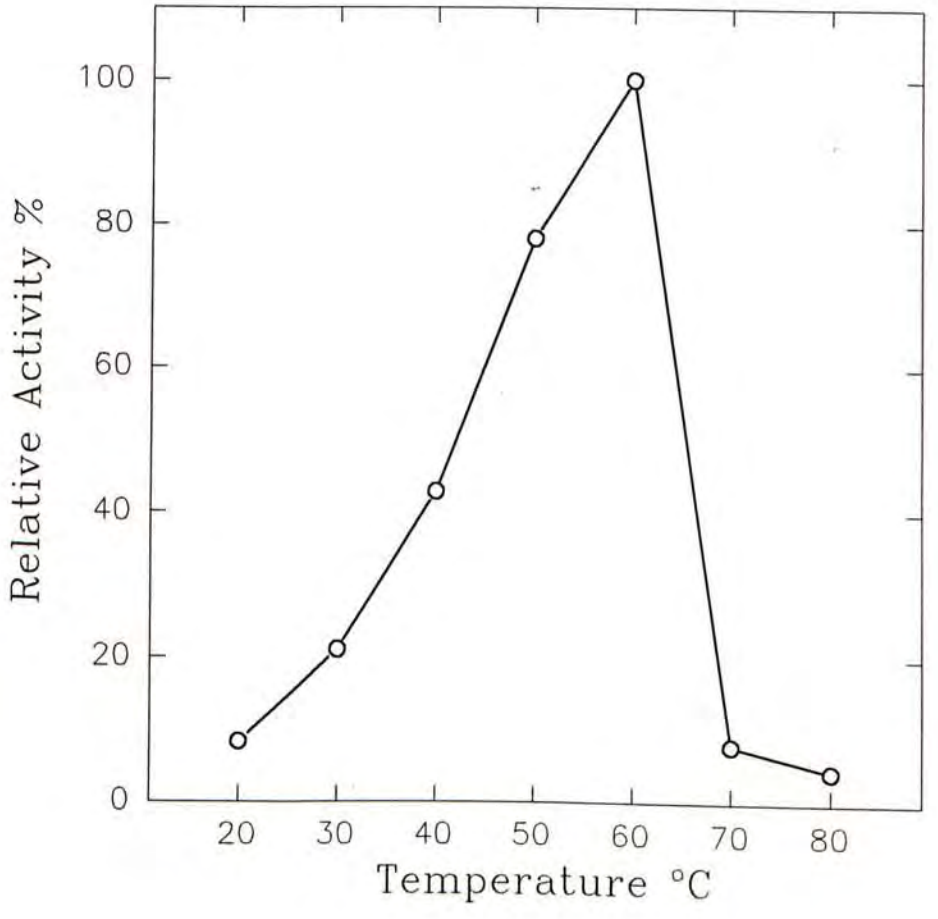

Figure 3.7 Effect of Temperature on the Activity of Extracellular ß-Xylosidase. Culture supernatants were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPX at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 60°C.

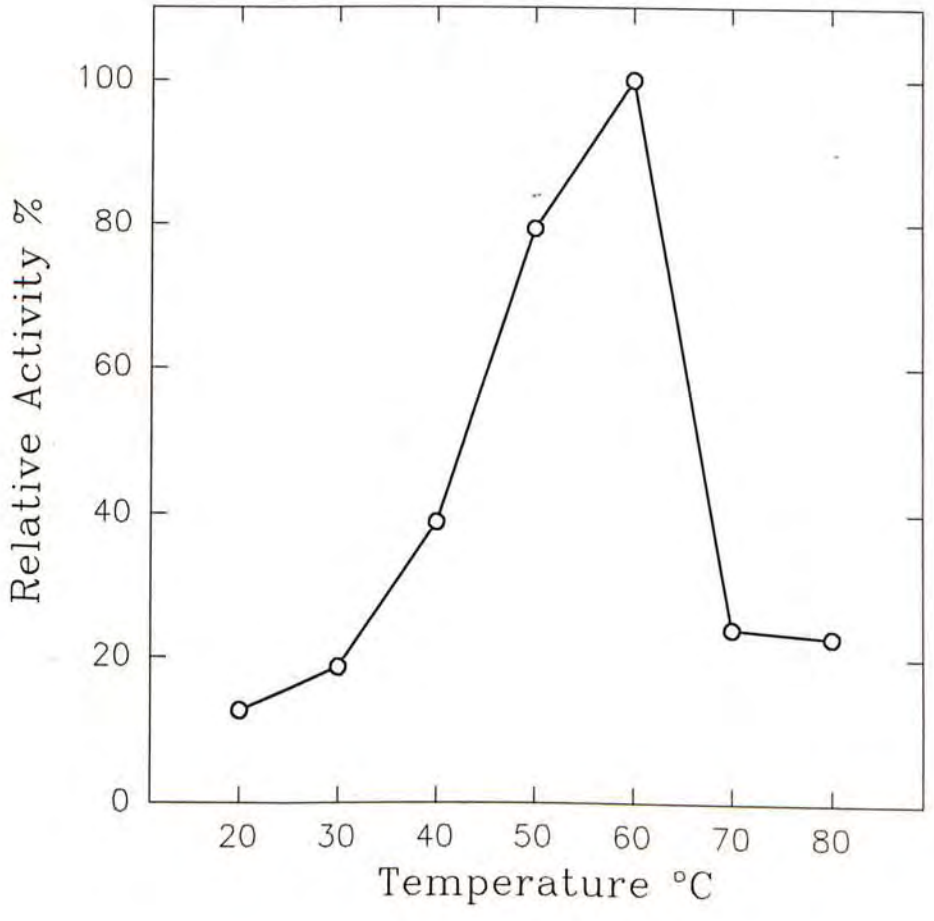

Figure 3.8 Effect of Temperature on the Activity of Cell-associated ß-Xylosidase. Enzyme extracts were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPX at various temperatures for 30 minutes. The amount of product released were determined. Highest activity (100%) was observed at 60°C.

Figure 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, and 3.15 showed the plots of the amounts of products released from substrates by enzymic activities over 30 minutes. For ß-glucosidases (Figure 3.11, 3.12) and ß-xylosidases (Figure 3.14, 3.15), enzymic activities were linear with time over 30 minutes. Therefore, for all later experiments, 15 minutes was selected as the assay time for ß-glucosidases and ß-xylosidases. With reference to Figure 3.9 and 3.10, the reducing sugar released from the activity of exoglucanase and endoglucanase increased with time within the first 15 minutes and then seemed to level-off afterwards. Hence, for exoglucanase and endoglucanase, 15 minutes was selected as the assay time. In the case of ß-xylanase, the amount of reducing sugar released increased tremendously within the first 5 minutes and then levelled-off. After 25 minutes, very little increase in degradation product was observed. This phenomenon was probably related to the physical nature of the substrate, birchwood xylan, used in the assay. For this reason, 20 minutes was selected as the assay time of ß-xylanase in subsequent studies.

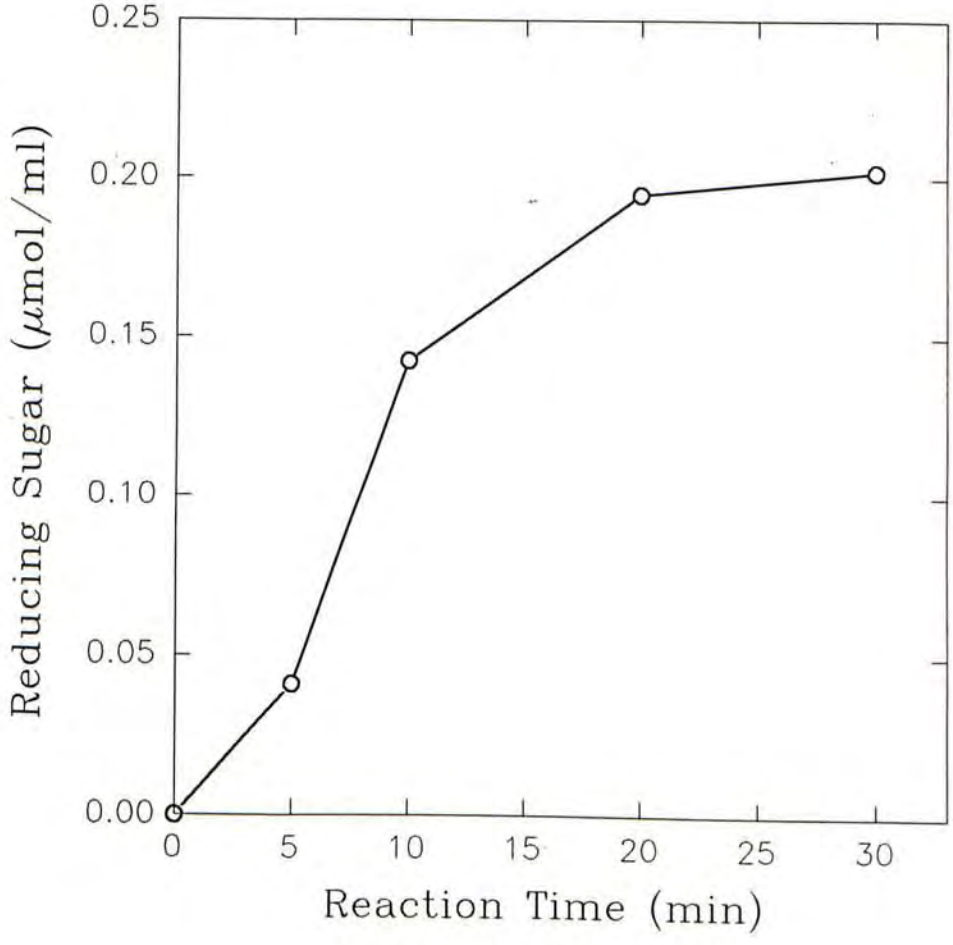

Figure 3.9 Release of Reducing Sugar (Glucose Equivalents) by Exoglucanase Activity over Time Course. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with Avicel at 40°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

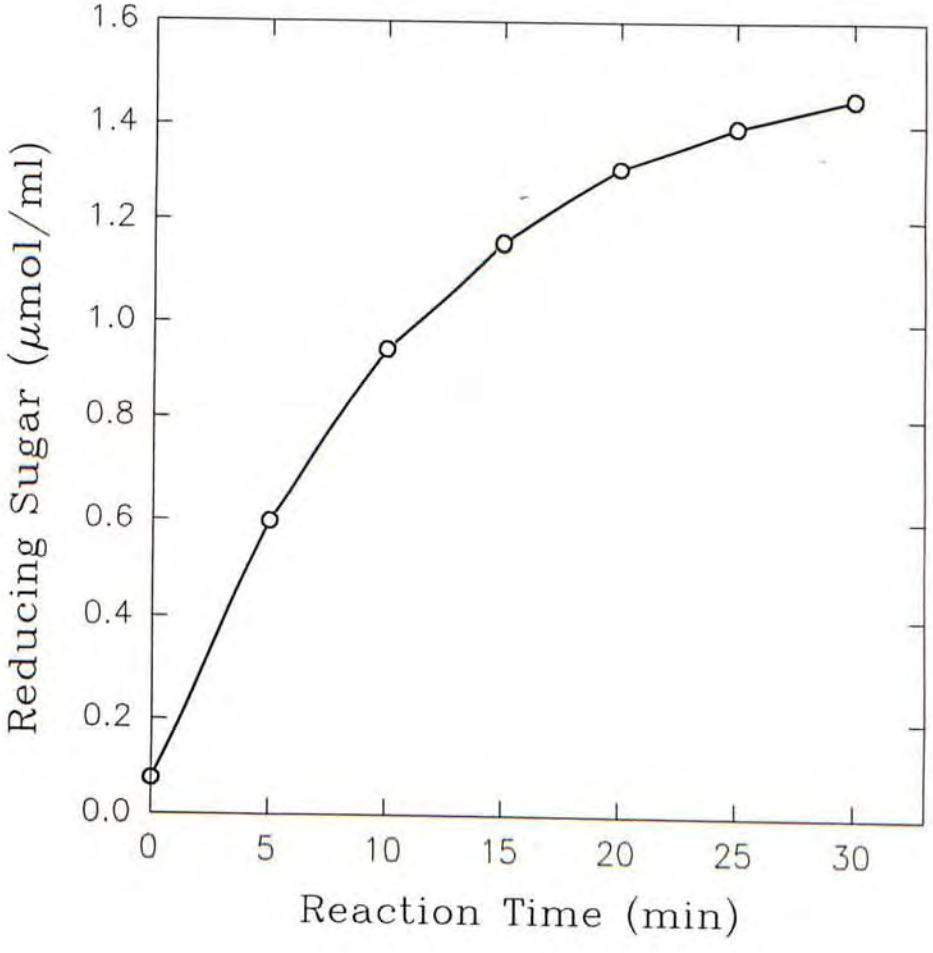

Figure 3.10 Release of Reducing Sugar (Glucose Equivalents) by Endoglucanase Activity over Time Course. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with CMC at 50°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

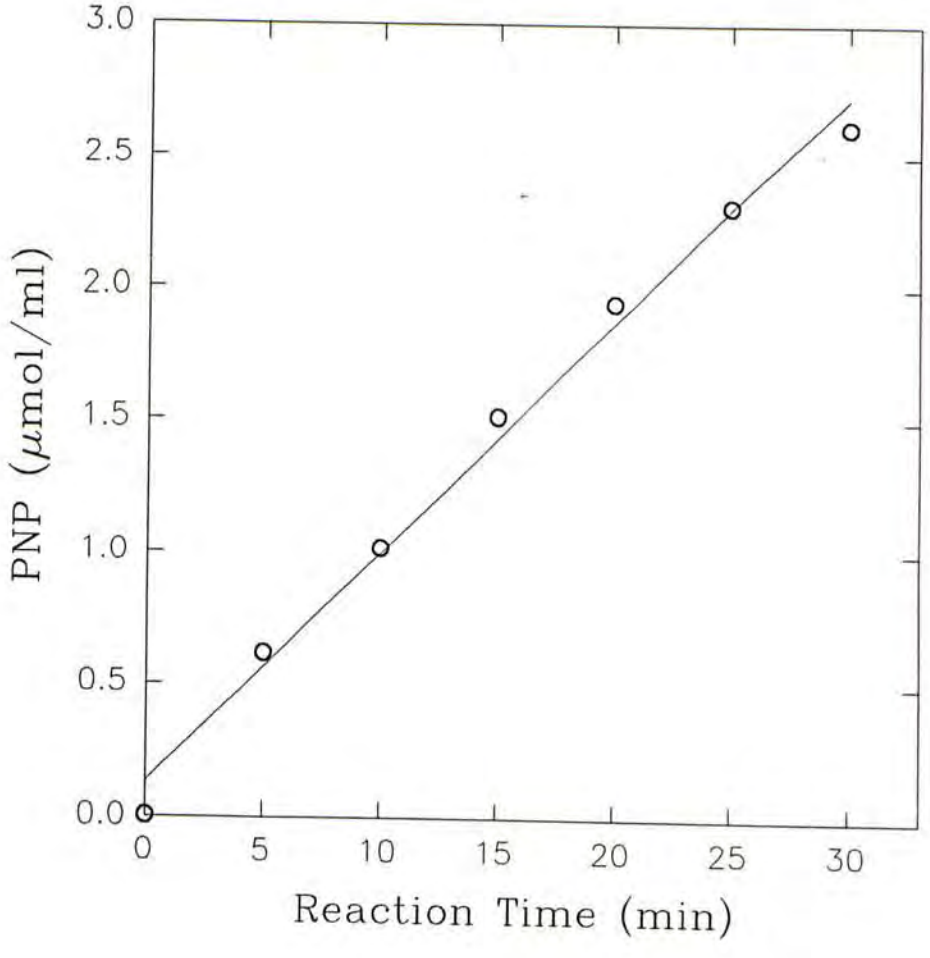

Figure 3.11 Release of PNP by Extracellular ß-Glucosidase Activity over Time Course. Culture supernatants were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH6, with PNPG at 50°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

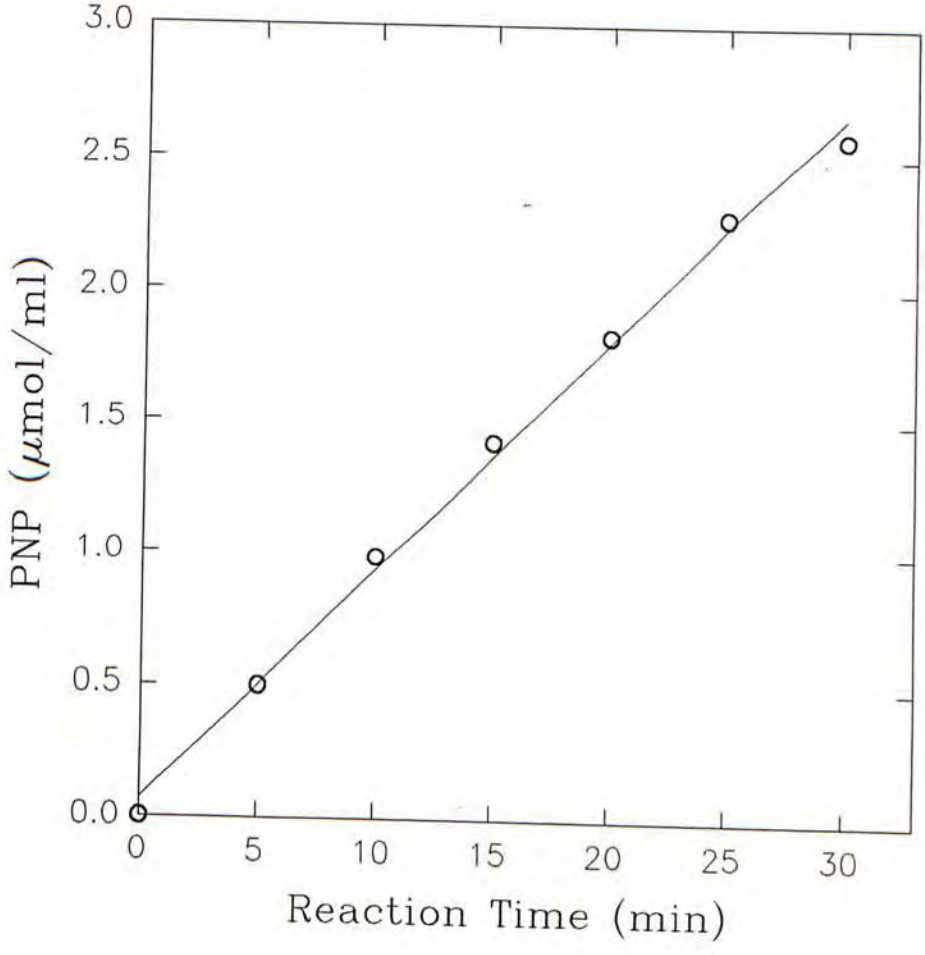

Figure 3.12 Release of PNP by Cell-associated ß-Glucosidase Activity over Time Course. Enzyme extracts were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPG at 50°C. Assays were terminated at various time intervals and the amount of product released plottted against time.

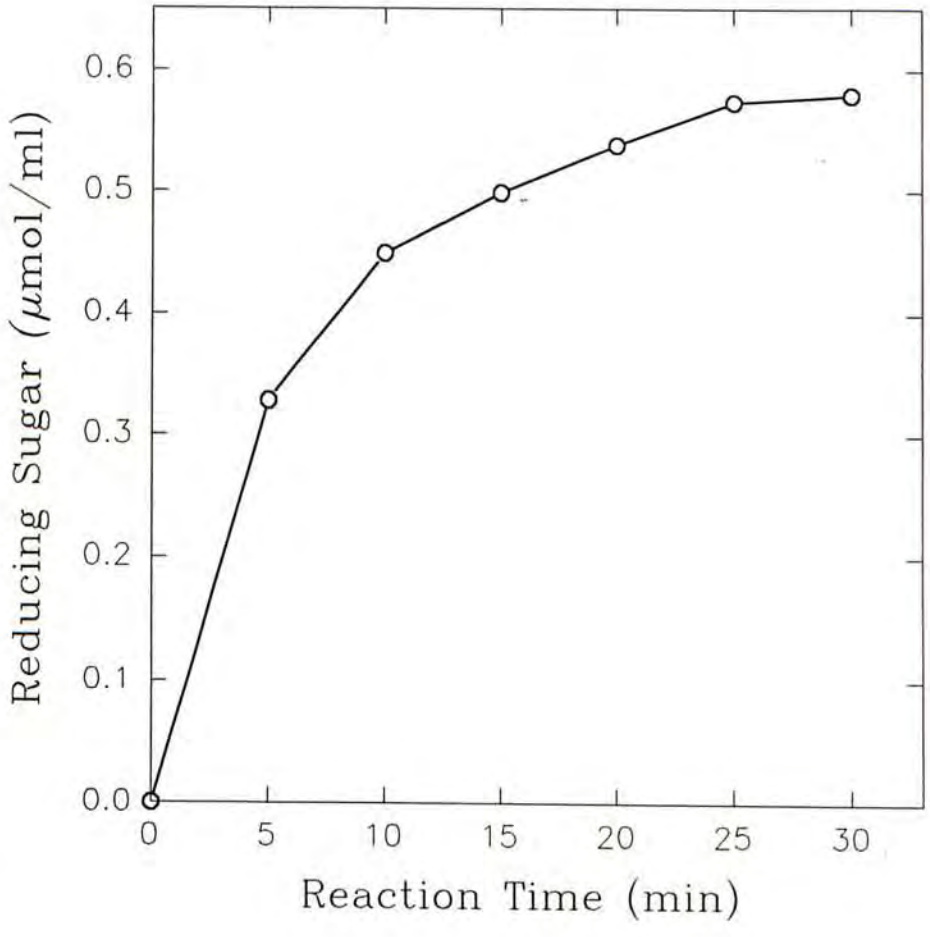

Figure 3.13 Release of Reducing Sugar (Xylose Equivalents) by ß-Xylanase Activity over Time Course. Culture supernatants were incubated in 50 mM phosphate buffer, pH 6, with birchwood xylan at 40°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

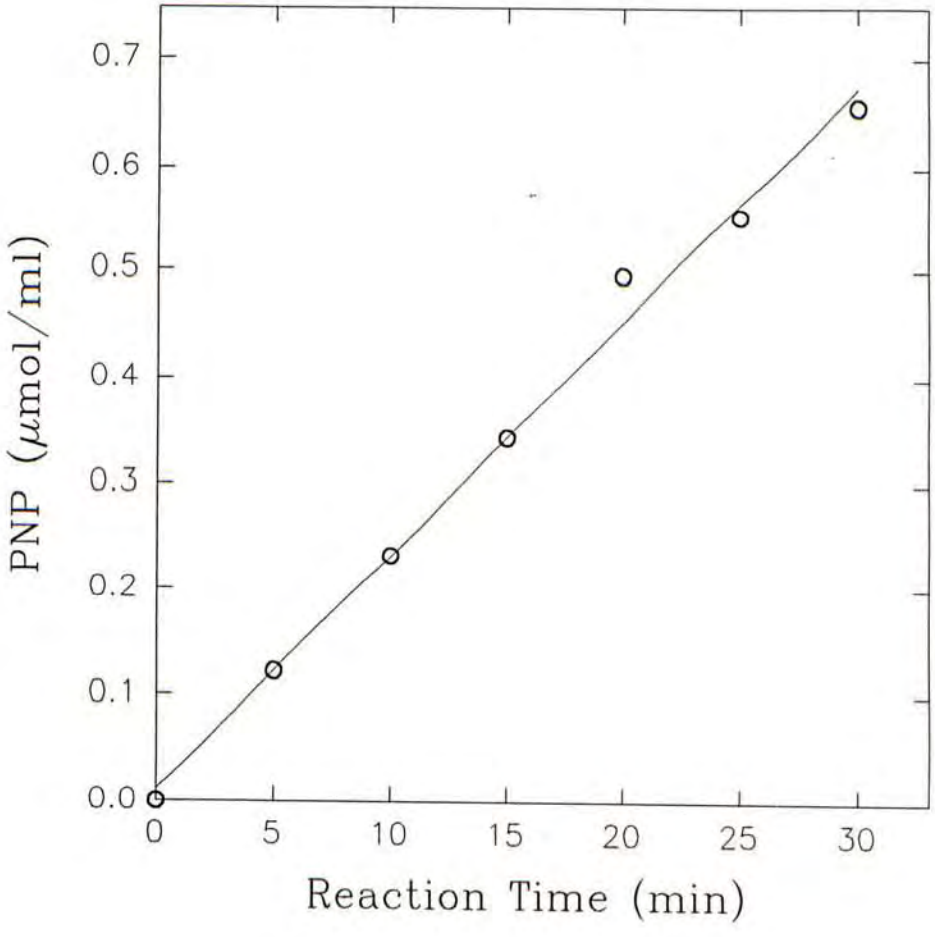

Figure 3.14 Release of PNP by Extracellular ß-Xylosidase Activity over Time Course. Culture supernatants were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPX at 50°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

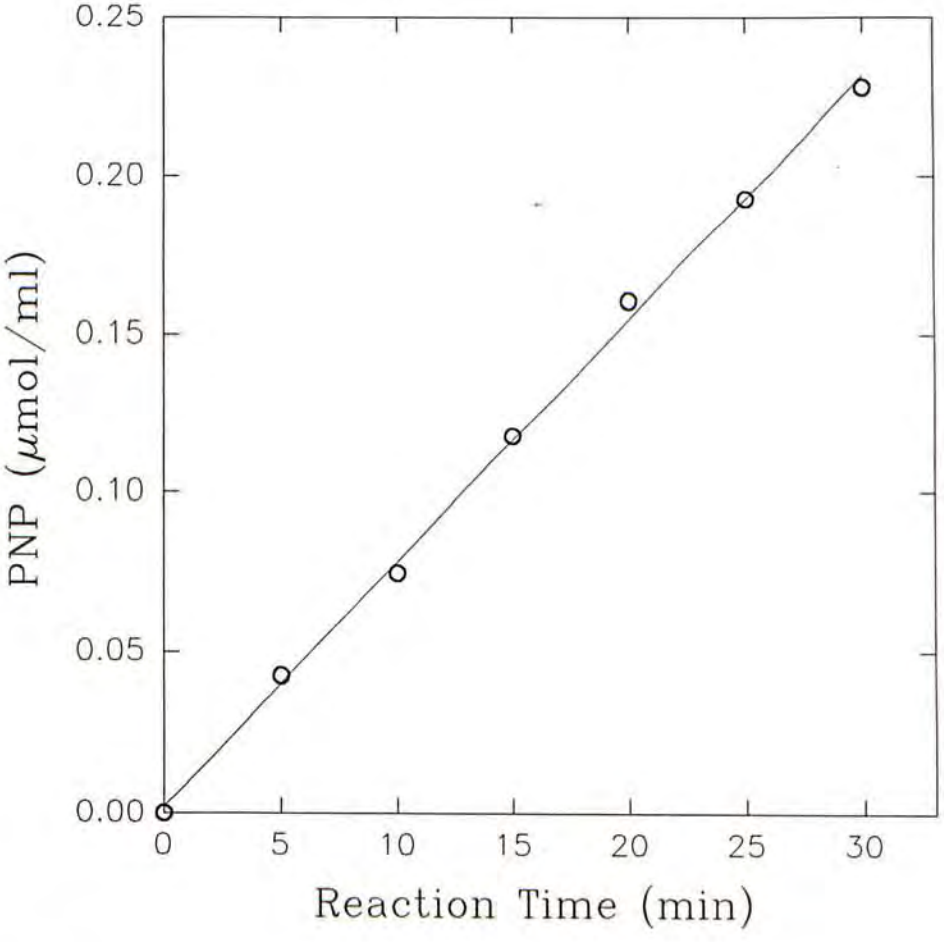

Figure 3.15 Release of PNP by Cell-associated ß-Xylosidase Activity over Time Course. Enzyme extracts were incubated in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4), pH 6, with PNPX at 50°C. Assays were terminated at various time intervals and the amount of product released plotted against time.

3.3 Determination of the Optimal pH for Enzyme Reaction

Figures 3.16, 3.17, 3.18, 3.19, 3.20, 3.21 and 3.22 show the effect of pH on the activity of exoglucanase, endoglucanase, extracellular and cell-associated ß-glucosidases, ß-xylanase, extracellular and cell-associated ß-xylosidases, respectively. The pH-optima for the enzymes were 7.4, 7.0, 5.4, 5.8, 7.0, 5.8 and 5.8 respectively. Extracellular ß-glucosidase, extracellular and cell-associated ß-xylosidases were active over a broad pH range and 80% of the maximum activity observed was retained within the pH range 5.0 to 6.6.

A summary of the conditions determined for optimum enzyme activity for use in later experiments is given in Table 3.1.

Table 3.1 Summary of the Optimum Conditions for the Assay of Cellulolytic and Xylanolytic Enzymes of *F. velutipes*

Enzyme	Temperature(°C)	pH	Duration(min)
Exoglucanase	40	7.4	15
Endoglucanase	50	7.0	15
Extracellular ß-glucosidase	50	5.4	15
Cell-associated ß-glucosidase	50	5.8	15
ß-Xylanase	40	7.0	20
Extracellular ß-xylosidase	50	5.8	15
Cell-associated ß-xylosidase	50	5.8	15

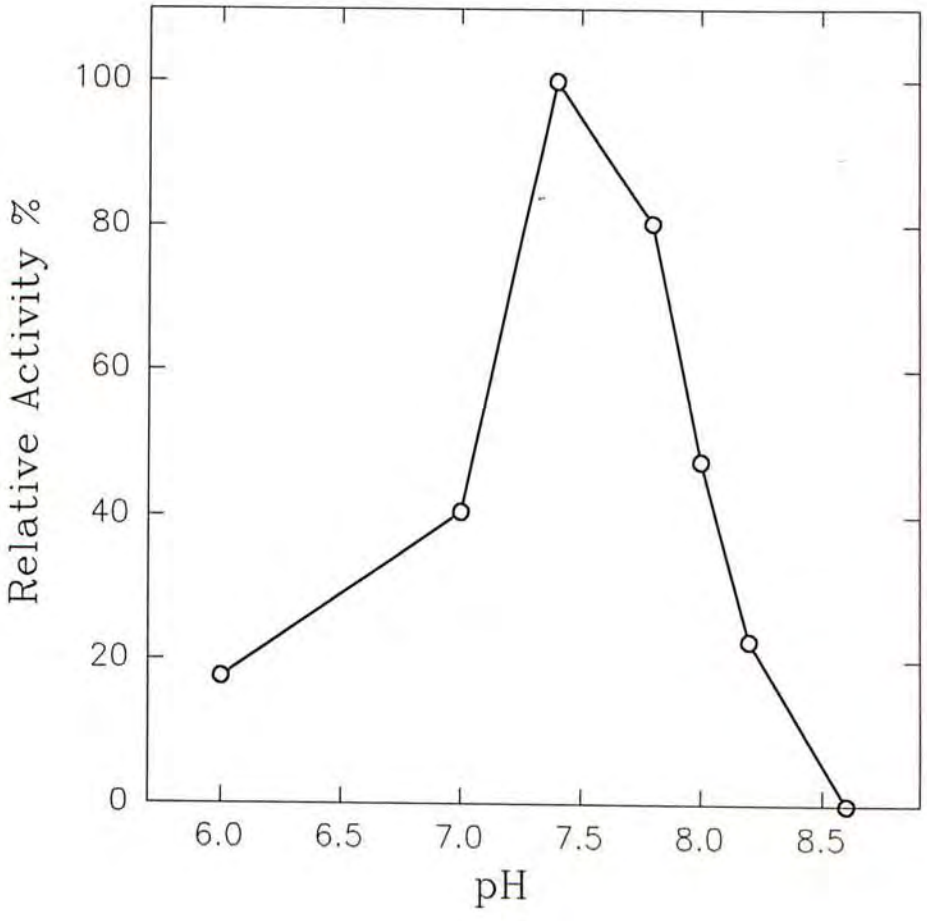

Figure 3.16 Effect of pH on the Activity of Exoglucanase. Culture supernatants were incubated with Avicel in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) adjusted to different pH at 40°C for 15 minutes. The amount of product released were determined. Highest activity (100%) was observed at pH 7.4.

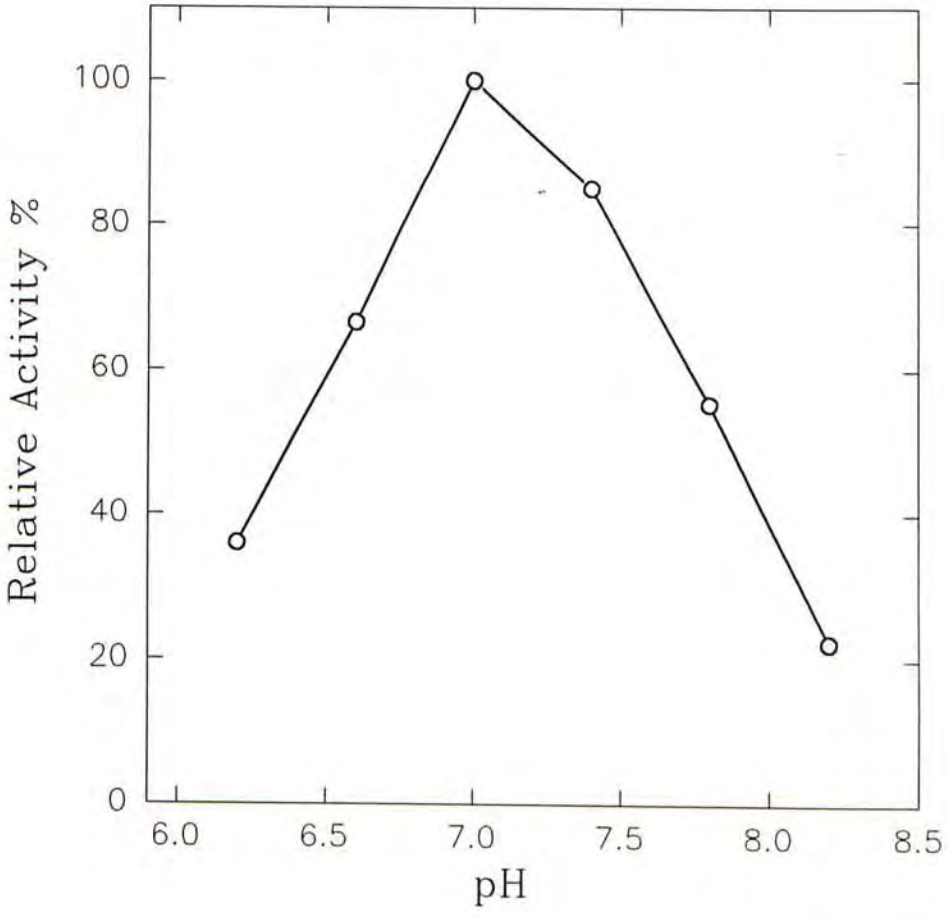

Figure 3.17 Effect of pH on the Activity of Endoglucanase. Culture supernatants were incubated with CMC in citrate-phosphate buffer (0.1M citric acid, 0.2M Na_2HPO_4) adjusted to different pH values at 50°C for 15 minutes. The amount of product released were derermined. Highest activity (100%) was observed at pH 7.0.

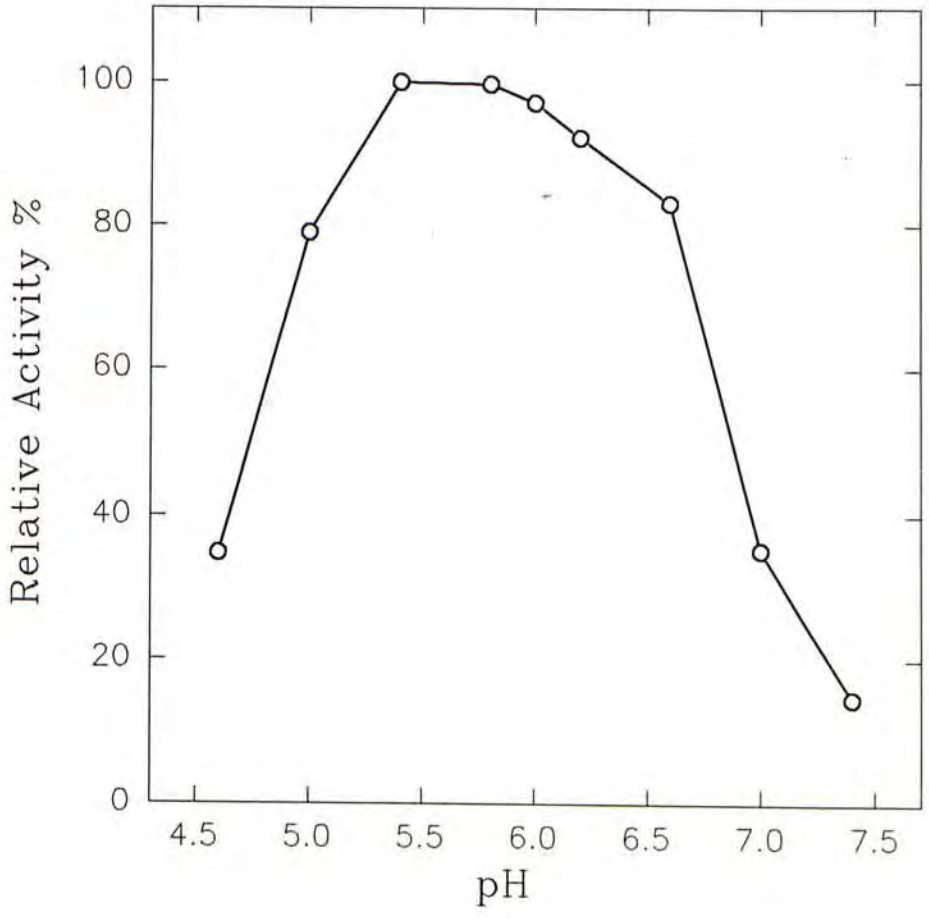

Figure 3.18 Effect of pH on the Activity of Extracellular ß-Glucosidase. Culture supernatants were incubated with PNPG in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) adjusted to different pH values at 50°C for 15 minutes. The amount of products released were determined. Highest activity (100%) was observed at pH 5.4.

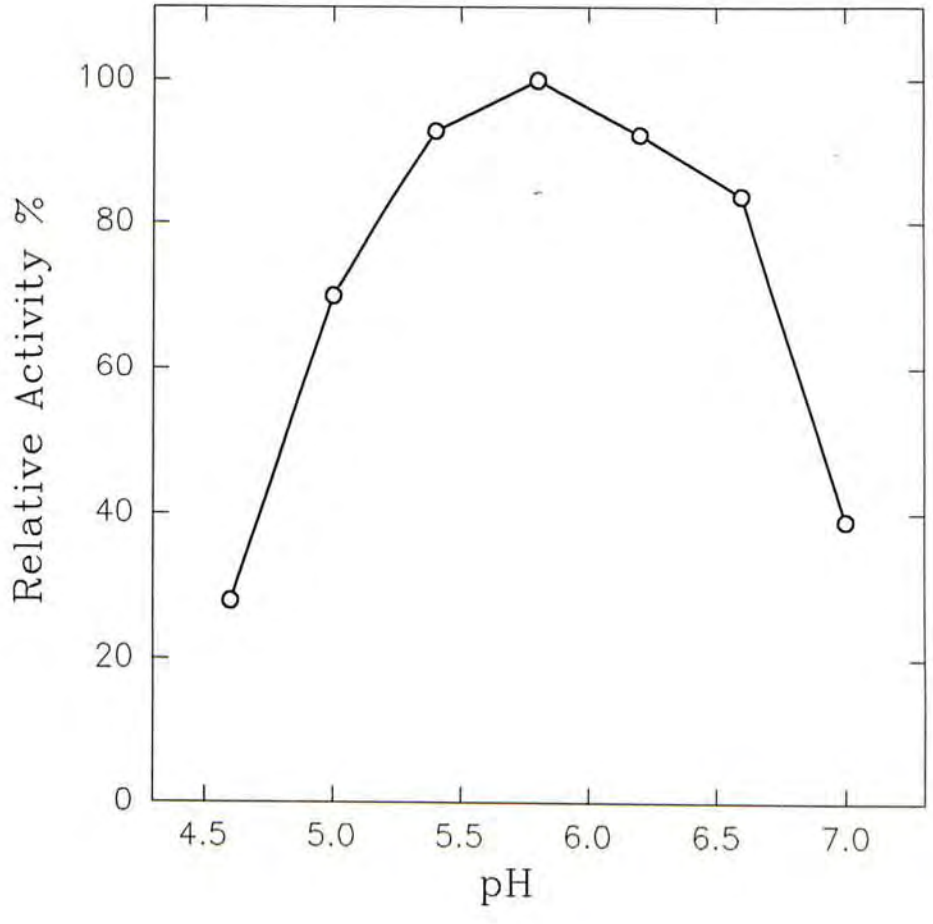

Figure 3.19 Effect of pH on the Activity of Cell-associated ß-Glucosidase. Enzyme extracts were incubated with PNPG in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4)) adjusted to different pH at 50°C for 15 minutes. The amount of product released were determined. Highest activity (100%) was observed at pH 5.8.

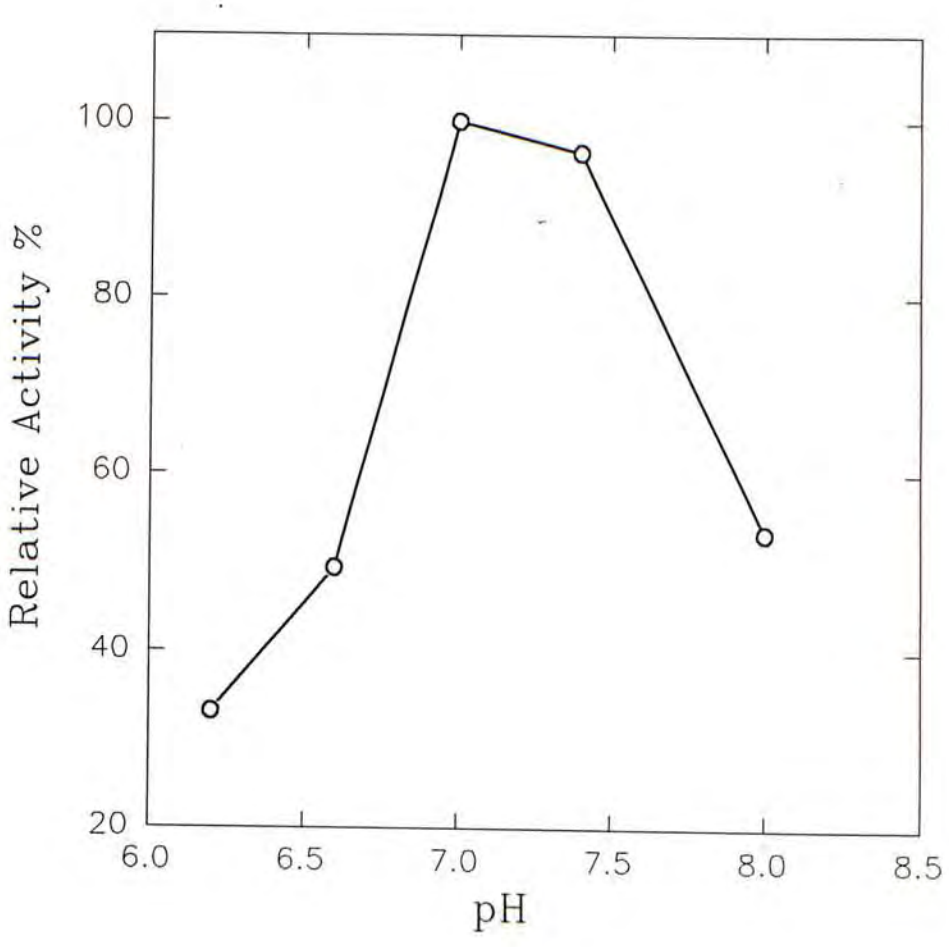

Figure 3.20 Effect of pH on the Activity of ß-Xylanase. Culture supernatants were incubated with birchwood xylan in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) adjusted to different pH values at 40°C for 20 minutes. The amount of product released were determined. Highest activity (100%) was observed at pH 7.0.

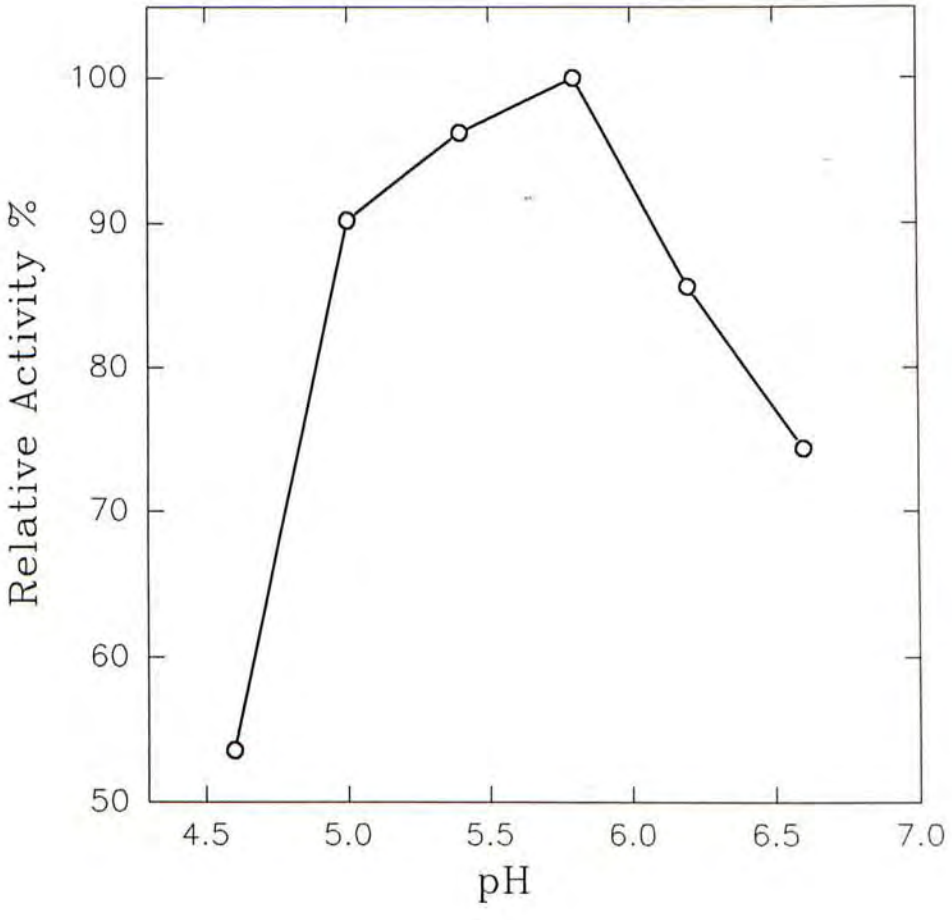

Figure 3.21 Effect of pH on the Activity of Extracellular ß-Xylosidase. Culture supernatants were incubated with PNPX in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) adjusted to different pH values at 50°C for 15 minutes. The amount of product released were determined. Highest activity (100%) was observed at pH 5.8.

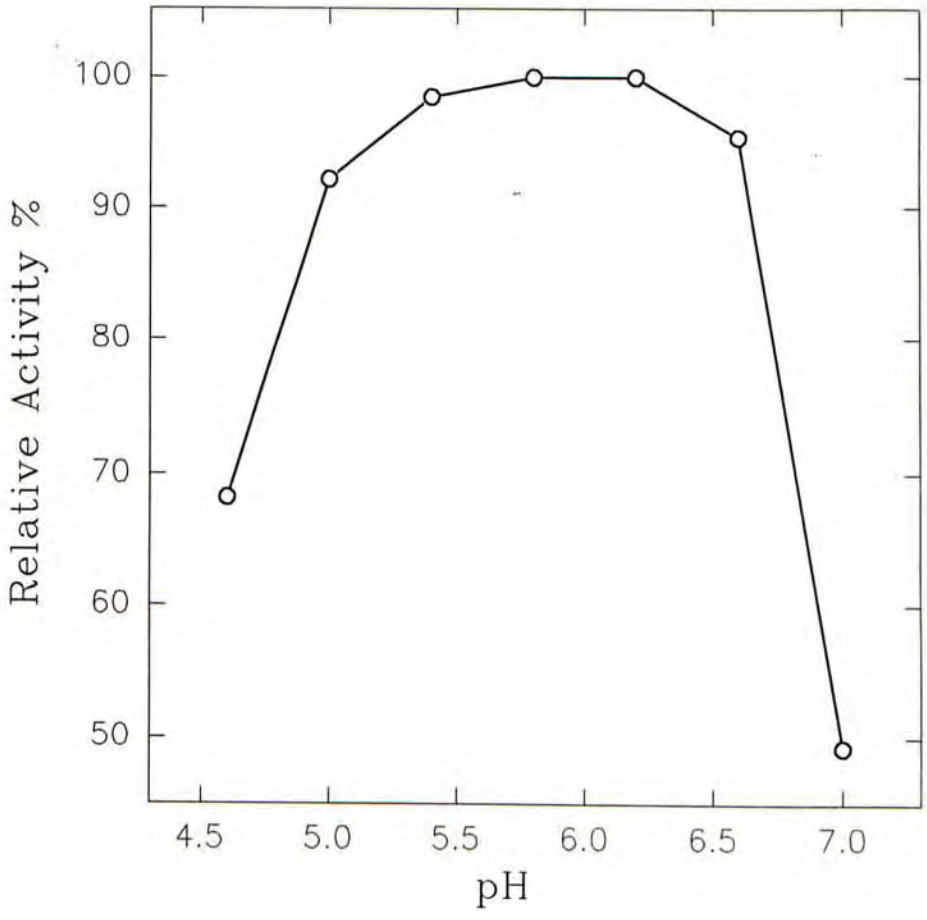

Figure 3.22 Effect of pH on the Activity of Cell-associated ß-Xylosidase. Enzyme extracts were incubated with PNPX in citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4) adjusted to different pH values at 50°C for 15 minutes. The amount of product released were determined. Highest activity (100%) was observed at pH 5.8.

3.4 Time Course Experiments on the Production of Cellulolytic and Hemicellulolytic Enzymes

3.4.1 Production of Cellulolytic Enzymes

Fungal cultures grown on DMS medium supplemented with Avicel were assayed for the production of exoglucanase, endoglucanase and ß-glucosidase over timecourses. The results are presented in Figures 3.23 and 3.24. Changes in the protein content and reducing sugar levels of culture supernatants are plotted in Figures 3.25 and 3.29.

Exoglucanase and endoglucanase activities could only be detected in the culture medium after day 15 (Figure 3.23). Activities of both enzymes then increased and fluctuated considerably during the remainder of the experimental period. Endoglucanase activity was considerably higher than exoglucanase and a 3-fold difference was observed at day 32.

In order to compare the production of extracellular and cell-associated ß-glucosidase, specific activity of both enzymes were plotted in Figure 3.24. Cell-associated ß-glucosidase was detected after 3 days of fungal growth while extracellular ß-glucosidase was detected after 5 days. The specific activity of extracellular ß-glucosidase increased steadily during day 3 to day 16. After day 16, the specific activity increased markedly up to a peak at day 23 and then decreased afterwards until the end of the experimental period. The specific activity of cell-associated ß-glucosidase increased initially up to day 10 after which a drop in activity occurred. From day 16 onwards, the specific activity stayed at a steady low level.

From day 0 to day 16 of the fungal cultivation, the extracellular protein content remained fairly stable but then dropped sharply from day 16 until day 26 before rising rapidly again to a peak at day 29.

Figure 3.29 showed the timecourse change of reducing sugar content (glucose equivalent) in the culture supernatant. Reducing sugar levels remained low with little variation observed throughout the experimental period.

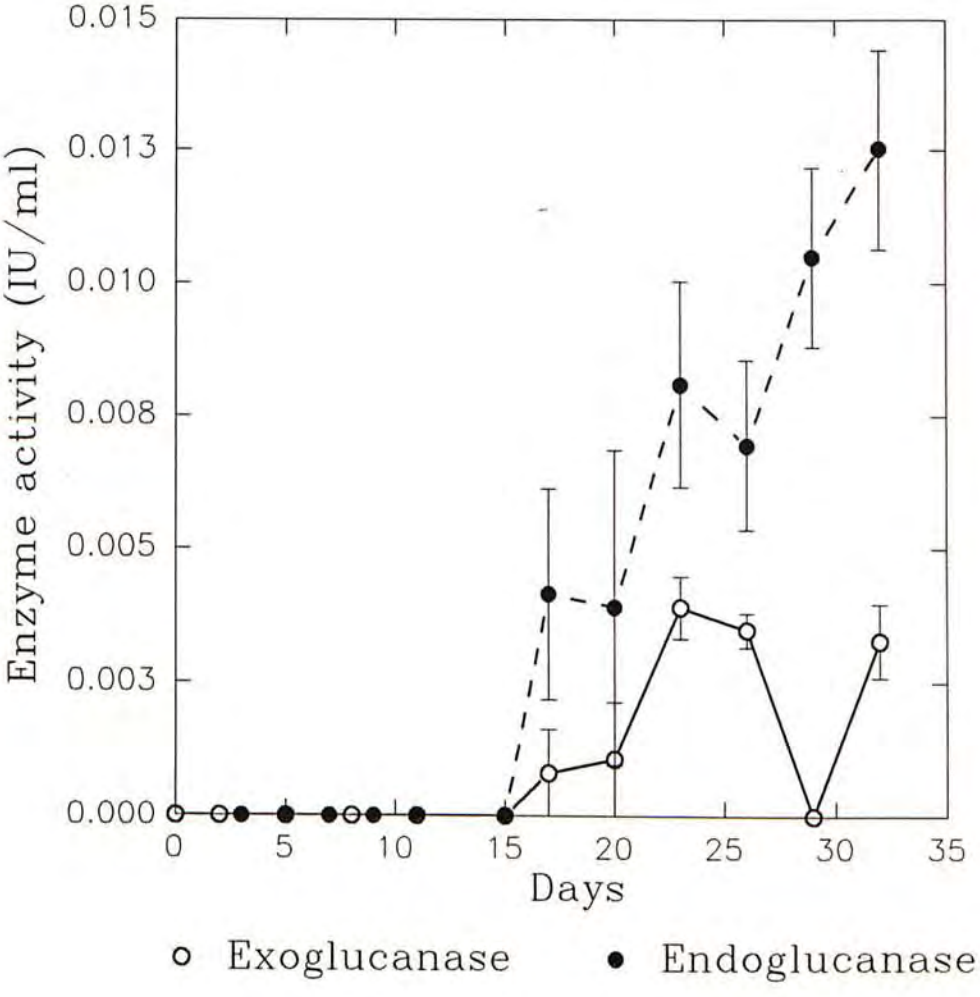

Figure 3.23 Production of Exoglucanase and Endoglucanase by *F. velutipes*. 1ml of mycelial homogenate was inoculated into 40ml Avicel-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the activity of cellulolytic enzymes determined. Error bars represent standard error of triplicate cultures.

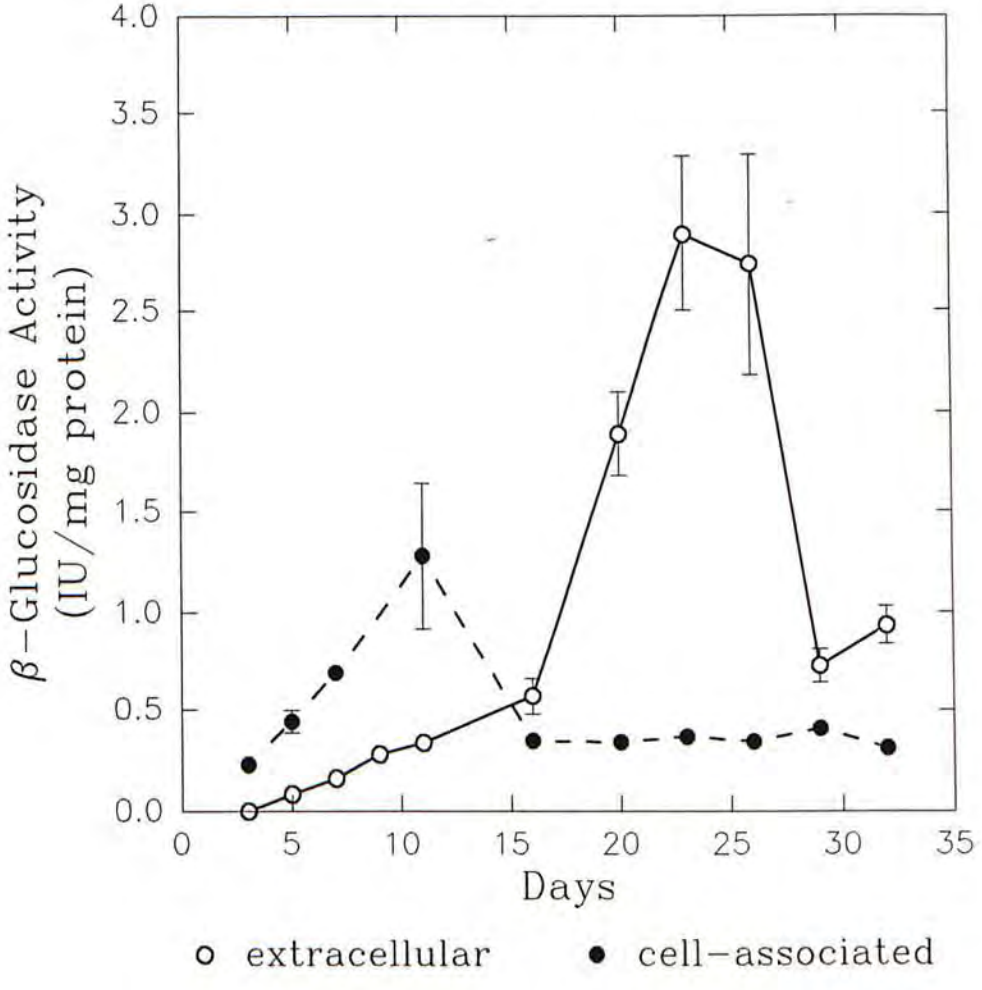

Figure 3.24 Production of Extracellular and Cell-associated ß-Glucosidases by *F. velutipes*. 1ml of mycelial homogenate was inoculated into 40ml Avicel-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the activity of ß-glucosidases determined. Error bars represent standard error of triplicate cultures.

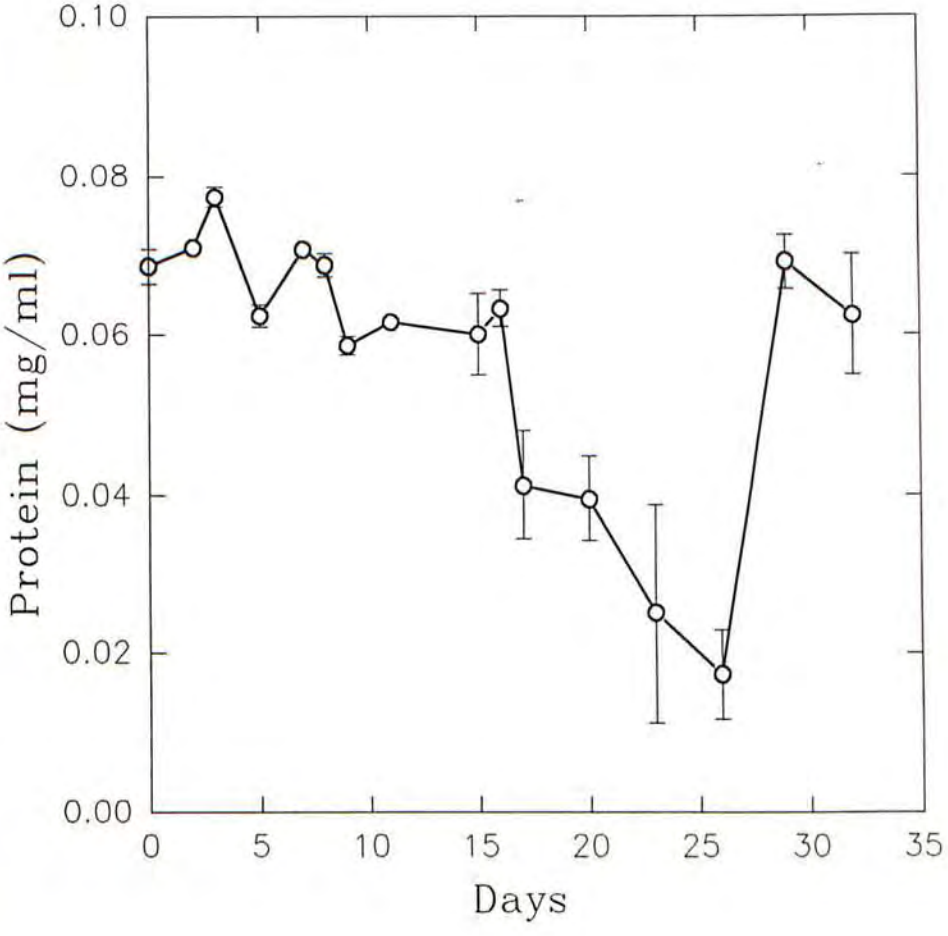

Figure 3.25 Changes in the Protein Content of Culture Supernatants of *F. velutipes* Grown on DMS Medium Supplemented with Avicel. 1ml of mycelial homogenate was inoculated into 40ml Avicel-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the protein content determined. Error bars represent standard error of triplicate cultures.

3.4.2 Production of Hemicellulolytic Enzymes

Fungal cultures grown on DMS medium supplemented with birchwood xylan were assayed for the production of ß-xylanase and ß-xylosidase over timecourses. The results are presented in Figures 3.26 and 3.27. Changes in the protein content and reducing sugar levels of culture supernatants are plotted in Figures 3.28 and 3.29.

As seen in Figure 3.26, a marked increase in ß-xylanase production was observed after 5 days of growth and continued up to about day 11. Enzyme production then levelled off and began to decrease after day 20. Production of ß-xylosidase showed a similar pattern. A continuous steady increase in enzyme production was observed with a slight decrease after day 20 (Figure 3.27).

To compare the production of extracellular and cell-associated ß-xylosidase, the specific activity of both enzymes were plotted in Figure 3.27. *F. velutipes* produced much more extracellular ß-xylosidase than cell-associated enzyme. The specific activity of cell-associated ß-xylosidase stayed at a steady low level with a slight increase during the period studied.

Changes in protein content in culture supernatants are shown in Figure 3.28. An insignificant change in extracellular protein content was observed during day 0 to day 16 after which a drop in protein levels occurred which correlated with a reduction and level-off in ß-xylanase and ß-xylosidase activities, respectively.

Figure 3.29 shows the timecourse change of reducing sugar content (xylose equivalent) in the culture supernatants. The reducing sugar levels increased tremendously from day 5 onwards due to the degradation of substrate (xylan) and peaked at day 11 followed by a gradual decrease in concentration as the products were metabolized.

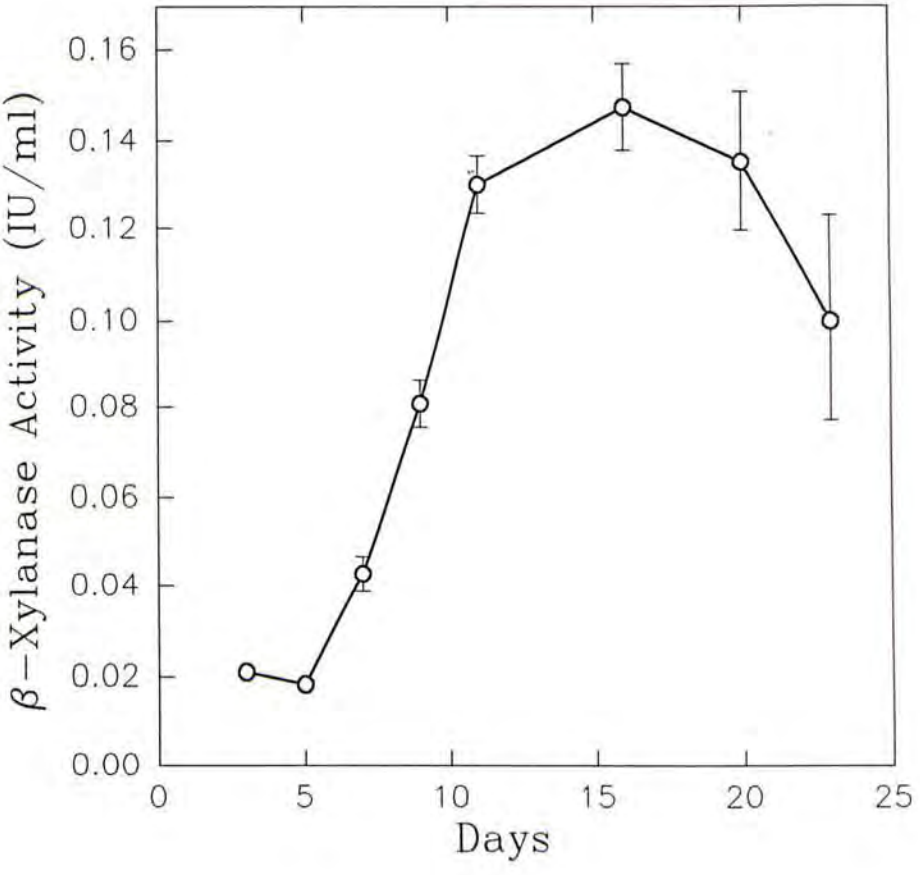

Figure 3.26 Production of ß-Xylanase by *F. velutipes*. 1ml of mycelial homogenate was inoculated into 40ml birchwood xylan-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the activity of ß-xylanase determined. Error bars represent the standard error of triplicate cultures.

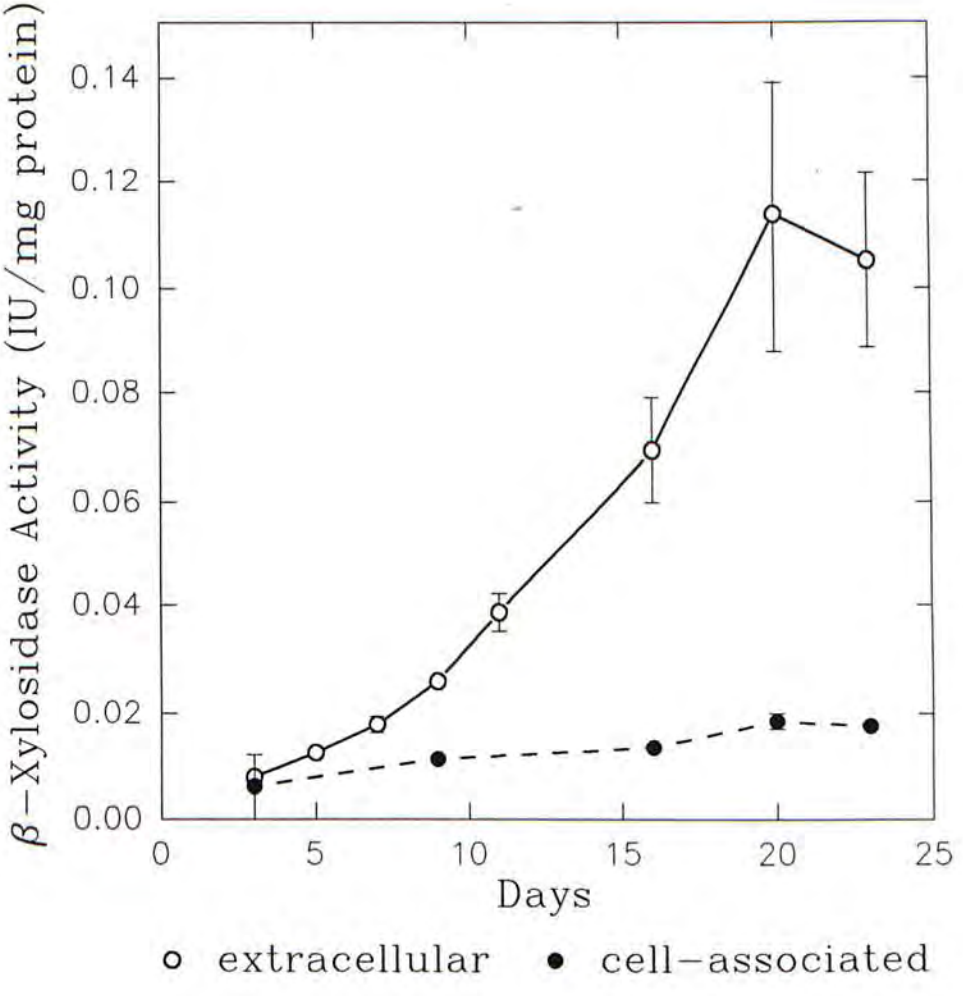

Figure 3.27 Production of Extracellular and Cell-associated ß-Xylosidase by *F. velutipes*. 1ml of mycelial homogenate was inoculated into 40ml birchwood xylan-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the activity of ß-xylosidases determined. Error bars represent the standard error of triplicate cultures.

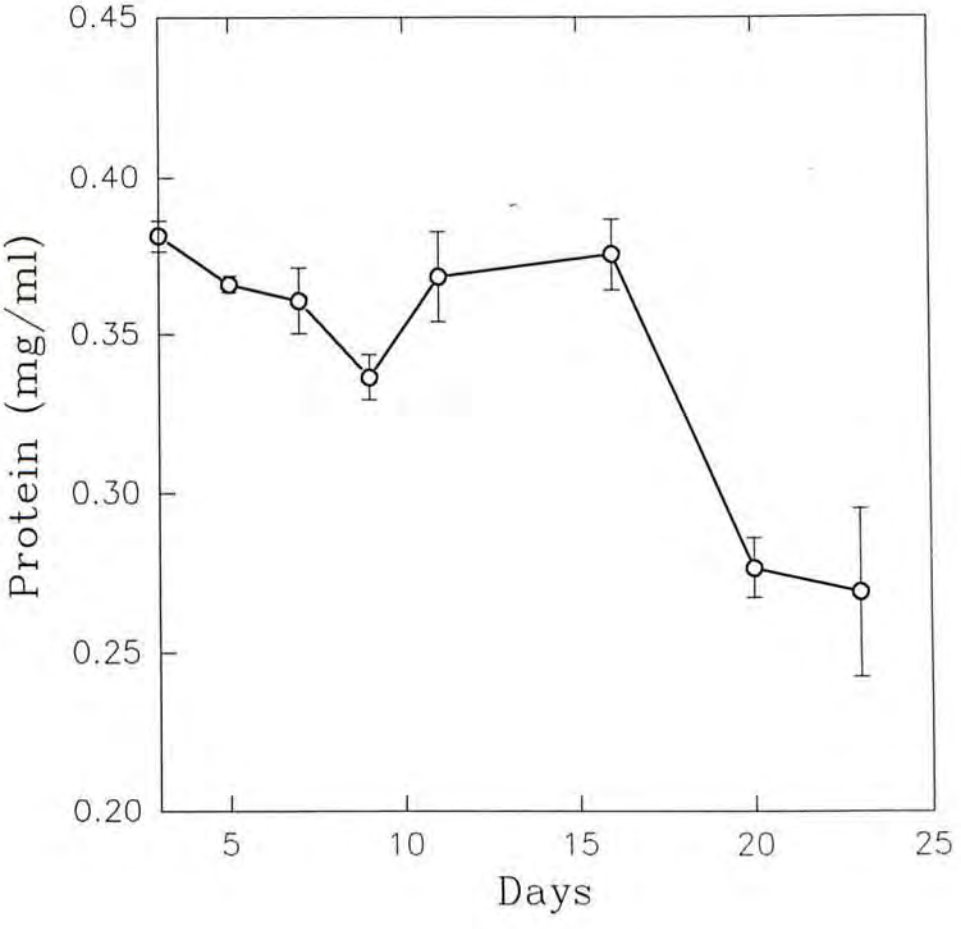

Figure 3.28 Changes in the Protein Content of Culture Supernatants of *F. velutipes* Grown on DMS Medium Supplemented with Birchwood Xylan. 1ml of mycelial homogenate was inoculated into 40ml birchwood xylan-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the protein content determined. Error bars represent the standard error of triplicate cultures.

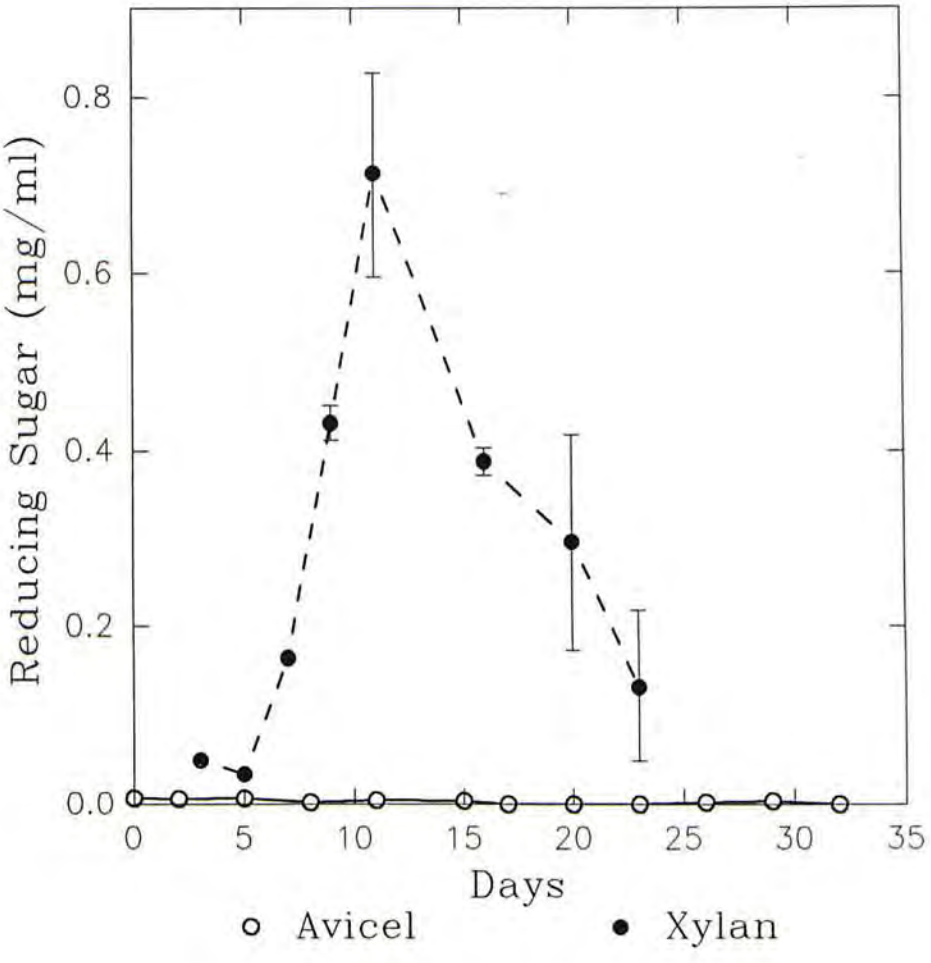

Figure 3.29 Changes in Reducing Sugar Levels in Culture Supernatants of *F. velutipes* Grown on DMS Medium Supplemented with Avicel or Birchwood Xylan. 1ml of mycelial homogenate was inoculated into 40ml Avicel- or birchwood xylan-supplemented (1% w/v) DMS medium and incubated at 25°C, 150rpm. Triplicate cultures were harvested at intervals and the amount of reducing sugar determined. Reducing sugar contents in Avicel cultures were measured as glucose equivalents while those in xylan cultures were measured as xylose equivalents.

3.5 Determination of Enzyme Induction Patterns

PDB-grown fungal pellets transferred to DMS media supplemented with substrates, Avicel, CMC, rice straw, filter paper and cotton wool were assayed for the production of cellulolytic and hemicellulolytic enzymes. The results are shown in Figures 3.30, 3.31, 3.32, 3.33 and 3.34. Changes in extracellular protein contents and reducing sugar levels in the induction flasks are plotted in Figures 3.35 and 3.36.

3.5.1 Induction of Exoglucanase Production

With reference to Figure 3.30, rice straw-supplemented culture gave the highest induction of exoglucanase production. Exoglucanase induction by rice straw was evident on about day 7 and enzyme activity rose rapidly to a peak at day 14. After day 14, a gradual reduction and a level-off in activity were observed. Lower levels of exoglucanase induction were observed when the fungus was grown on Avicel and CMC. Exoglucanase activities in media supplemented with these substrates were about half those detected with rice straw. Maximum yields of exoglucanase measured in cultures of *F. velutipes* grown on filter paper were about 0.02 IU/ml of culture medium which corresponded to only about 1/7, 1/3 and 1/4 of levels in culture medium containing rice straw, Avicel and CMC, respectively. Only traces of exoglucanase activity was detected in medium supplemented with cotton wool.

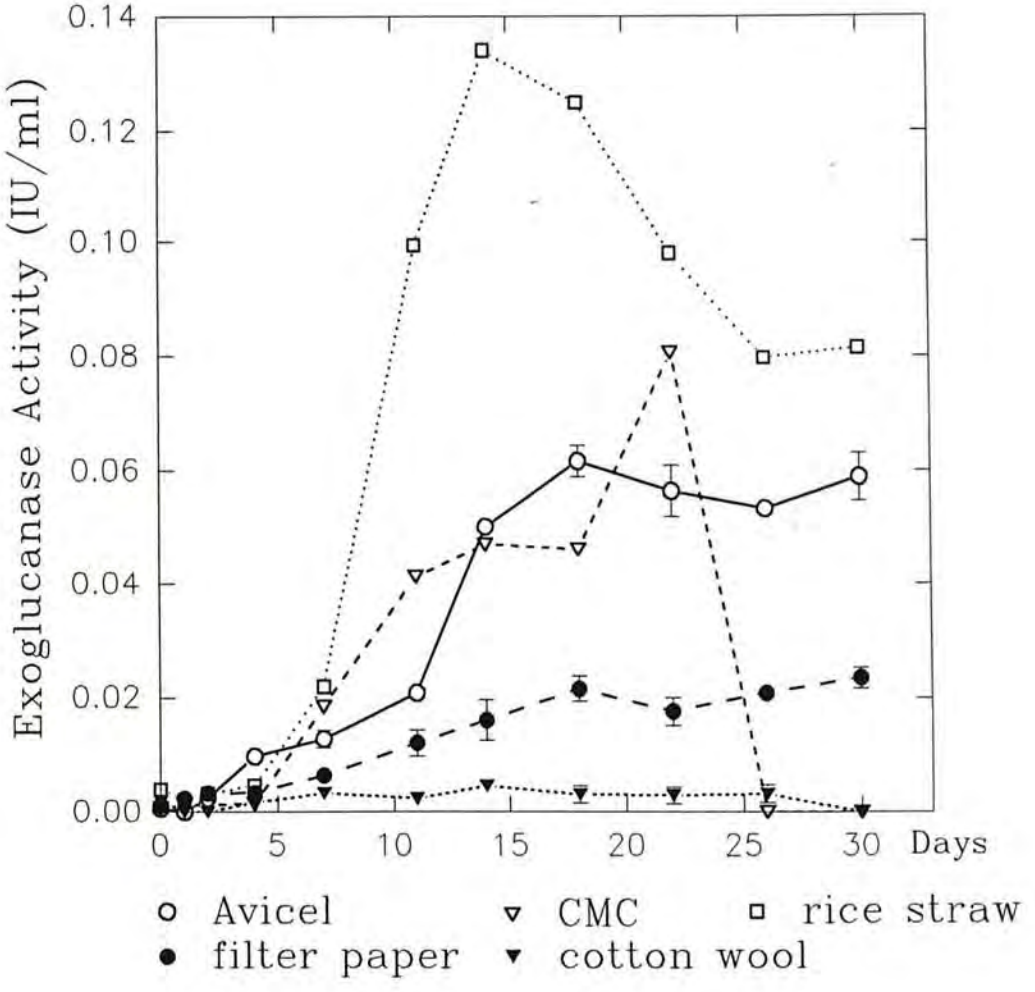

Figure 3.30 Production of Exoglucanase by *F. velutipes* in DMS Media Containing Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, Avicel, CMC, rice straw, filter paper or cotton wool. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for enzyme activity.

3.5.2 Induction of Endoglucanase Production

Figure 3.31 shows the production of endoglucanase by *F. velutipes* in DMS media containing different substrates. Avicel served as the best substrate for endoglucanase production and enzyme activity was evident at about day 4. Rice straw and filter paper ranked second and third most effective, respectively, although enzyme induction was not observed until day 7. Endoglucanase activities in culture media containing Avicel, rice straw and filter paper increased gradually up to day 18 and then levelled off. Surprisingly, CMC-supplemented medium only induced low levels of endoglucanase compared to Avicel-, rice straw- or filter paper-supplemented medium. In culture medium supplemented with cotton wool, little or no endoglucanase activity was detected.

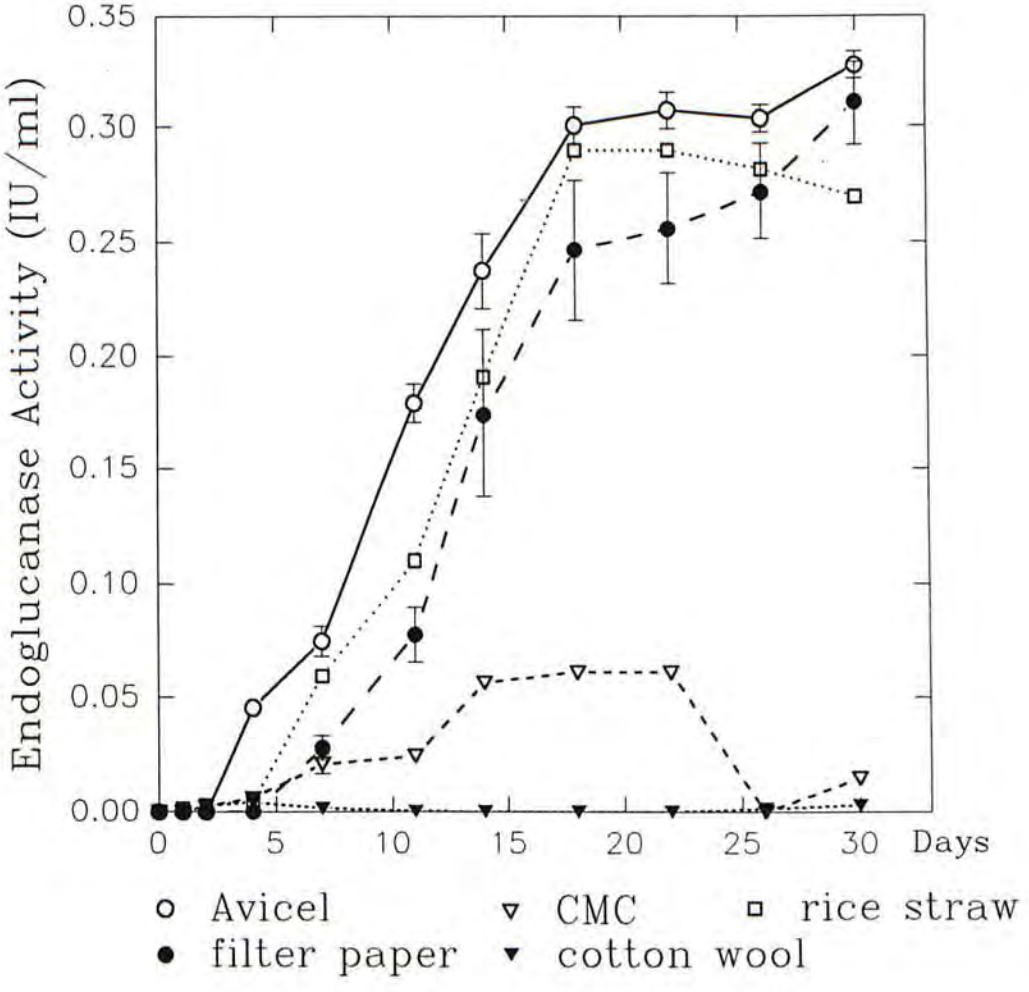

Figure 3.31 Production of Endoglucanase by *F. velutipes* in DMS Media Containing Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, Avicel, CMC, rice straw, filter paper or cotton wool. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for enzyme activity.

3.5.3 Induction of Extracellular ß-Glucosidase Production

Production of extracellular ß-glucosidase by *F. velutipes* in DMS media supplemented with different substrates is shown in Figure 3.32. Rice straw induced the highest levels of extracellular ß-glucosidase production followed by Avicel and filter paper. Enzyme activities in the culture medium containing rice straw, Avicel and filter paper rose continuously during the 30 day-experimental period. In contrast, extracellular ß-glucosidase production in cultures supplemented with CMC or cotton wool was only about 1/3, 1/3 and 1/5 of the maximum activities measured in Avicel, filter paper and rice straw media, respectively. Production of enzyme in CMC- and cotton wool-supplemented media remained at a steady low level throughout the experimental period.

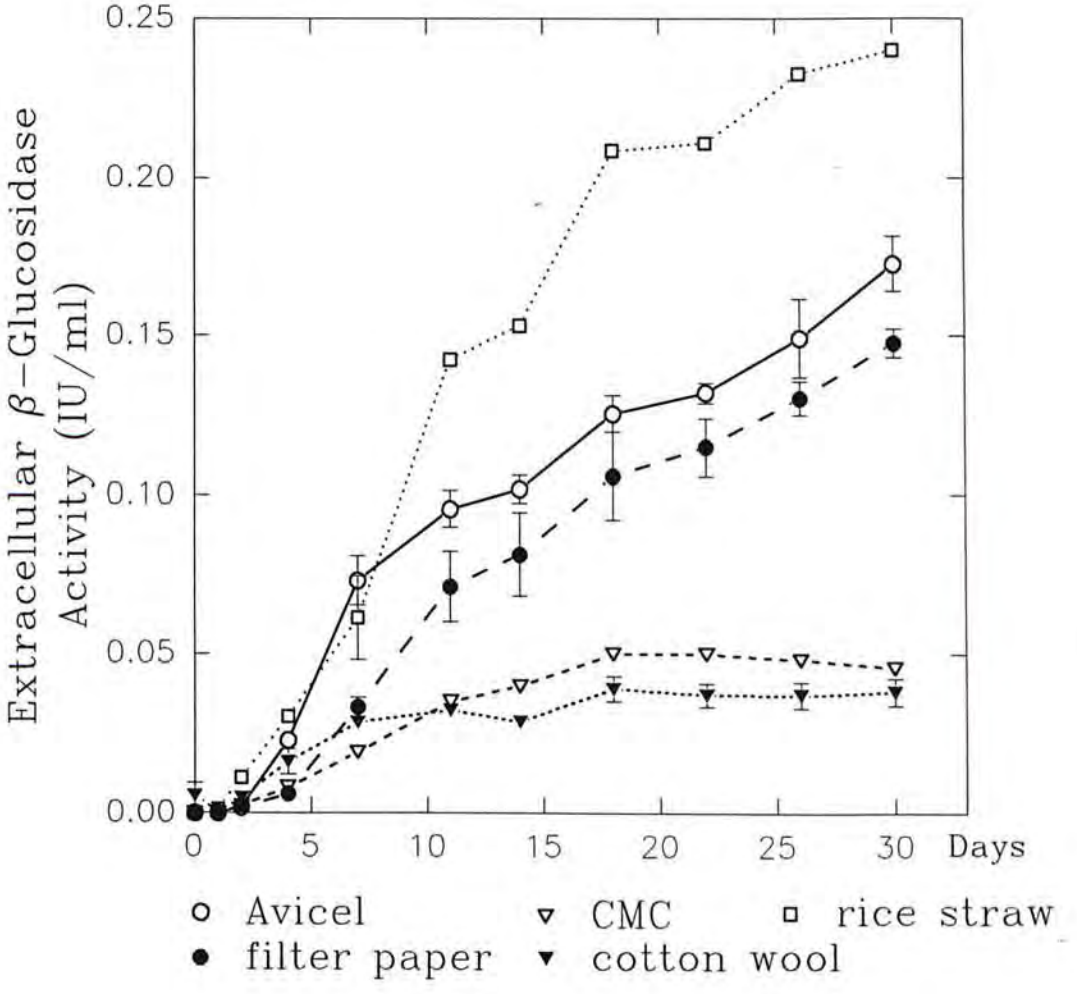

Figure 3.32 Production of Extracellular ß-Glucosidase by *F. velutipes* in DMS Media Containing Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, Avicel, CMC, rice straw, filter paper or cotton wool. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for enzyme activity.

3.5.4 Induction of ß-Xylanase Production

Fungal pellets transferred to DMS media containing rice straw or birchwood xylan were assayed for the production of ß-xylanase and the results shown in Figure 3.33.

Compared to birchwood xylan, rice straw was much more effective in inducing ß-xylanase production. Induction was observed after day 4 with enzyme activity increasing rapidly to a peak at day 18 followed by a gradual decrease. Only low levels of ß-xylanase activity was detected in birchwood xylan-supplemented media during the course of the experiment.

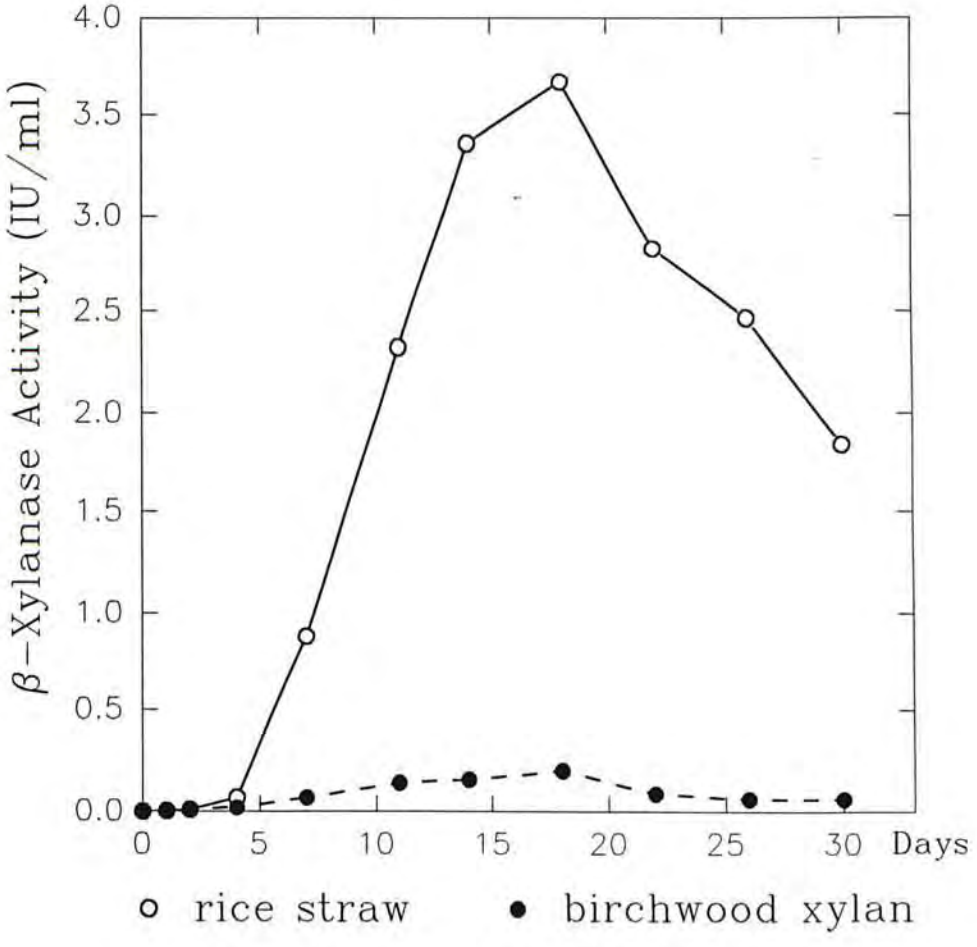

Figure 3.33 Production of ß-Xylanase by *F. velutipes* in DMS Media Containing Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, rice straw or birchwood xylan. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for enzyme activity.

3.5.5 Induction of Extracellular ß-Xylosidase Production

Production of extracellular ß-xylosidase was assayed in fungal cultures supplemented with birchwood xylan or rice straw and the results plotted in Figure 3.34.

Rice straw- and birchwood xylan-supplemented media were almost equally effective in inducing extracellular ß-xylosidase with slightly higher enzyme level observed in cultures supplemented with rice straw. Significant enzyme induction occurred in both media after about day 2, after which levels continued to increase over the 30-day experimental period. A levelling-off of enzyme activity was observed after day 26 in birchwood xylan-supplemented cultures.

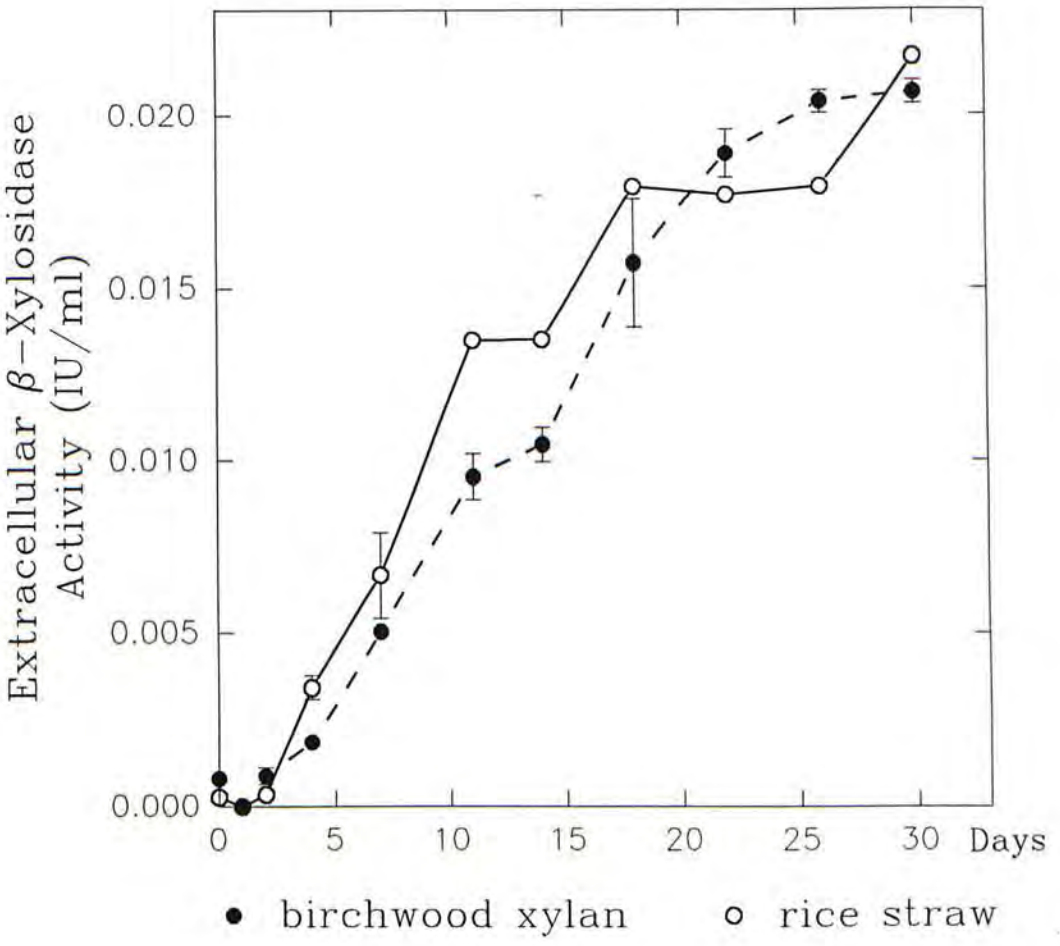

Figure 3.34 Production of Extracellular ß-Xylosidase by *F. velutipes* in DMS Media Containing Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, rice straw or birchwood xylan. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for enzyme activity.

3.5.6 Changes in Extracellular Protein Levels in DMS Media Supplemented with Different Substrates

Changes in extracellular protein levels in media supplemented with different substrates are shown in Figure 3.35. Highest protein levels were observed in cultures containing rice straw while little or no protein was detected in CMC- or cotton wool-containing cultures throughout the experiment. Slight increases in extracellular protein levels were observed after day 7 in culture flasks supplemented with Avicel and with filter paper.

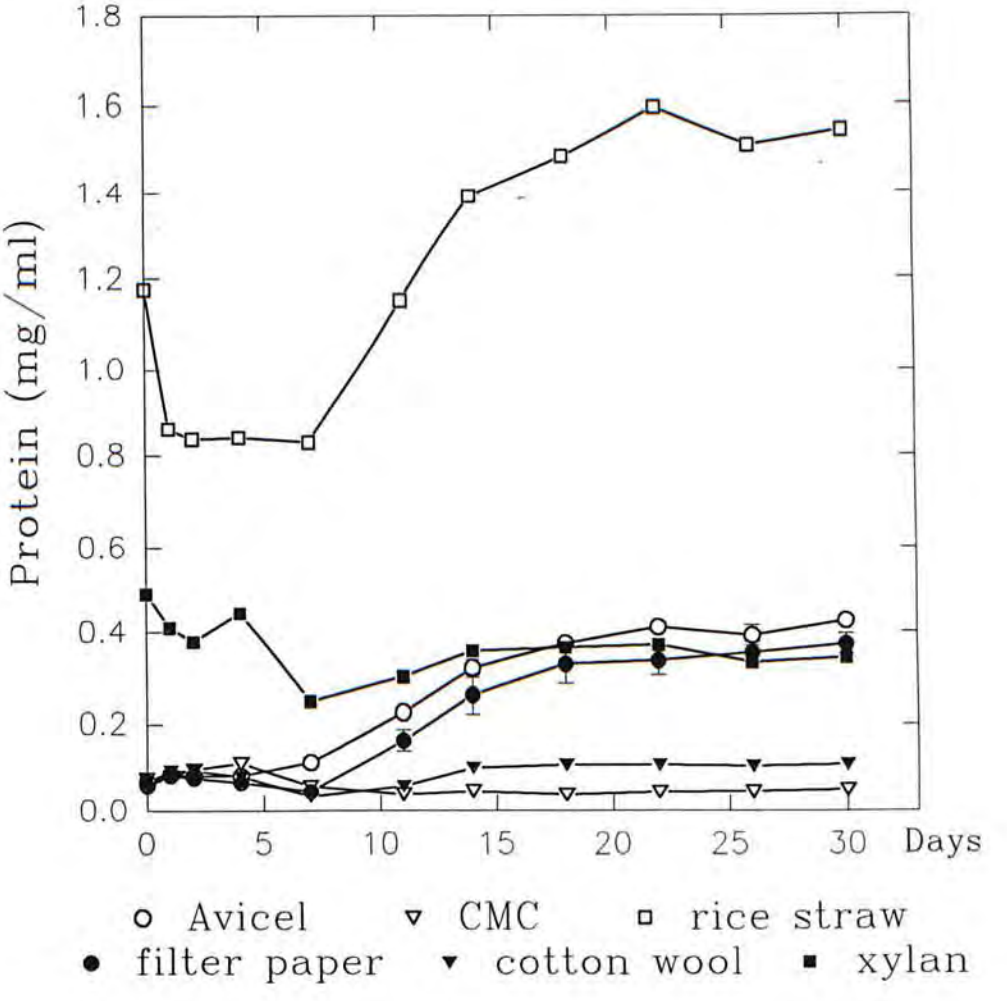

Figure 3.35 Changes in Extracellular Protein Levels in DMS Media Supplemented with Different Substrates. PDB-grown mycelial pellets were aseptically transferred to DMS medium supplemented with 1% (w/v) substrate, Avicel, CMC, rice straw, filter paper, cotton wool or birchwood xylan. Inoculated flasks were incubated at 25°C, 150rpm. Samples were taken at intervals from triplicate flasks and assayed for protein levels.

3.5.7 Changes in Reducing Sugar Levels in DMS Media Supplemented with Different Substrates

Figure 3.36 shows the changes in levels of reducing sugar in media supplemented with different substrates. Highest levels were recorded in xylan-supplemented cultures where reducing sugar in culture supernatants increased rapidly after day 1, peaked at day 11 and then decreased rapidly over the next 11 days. After day 22, reducing sugar levels remained at a constant low level to the end of the experimental period. The amount of reducing sugar in CMC-containing cultures increased after day 1 and then fluctuated only very slightly throughout the rest of the experimental period. Significant accumulation of reducing sugar in rice straw cultures did not occur until after day 11 after which a slight and steady increase was observed. Little or no reducing sugar was detected in cultures containing Avicel, filter paper or cotton wool.

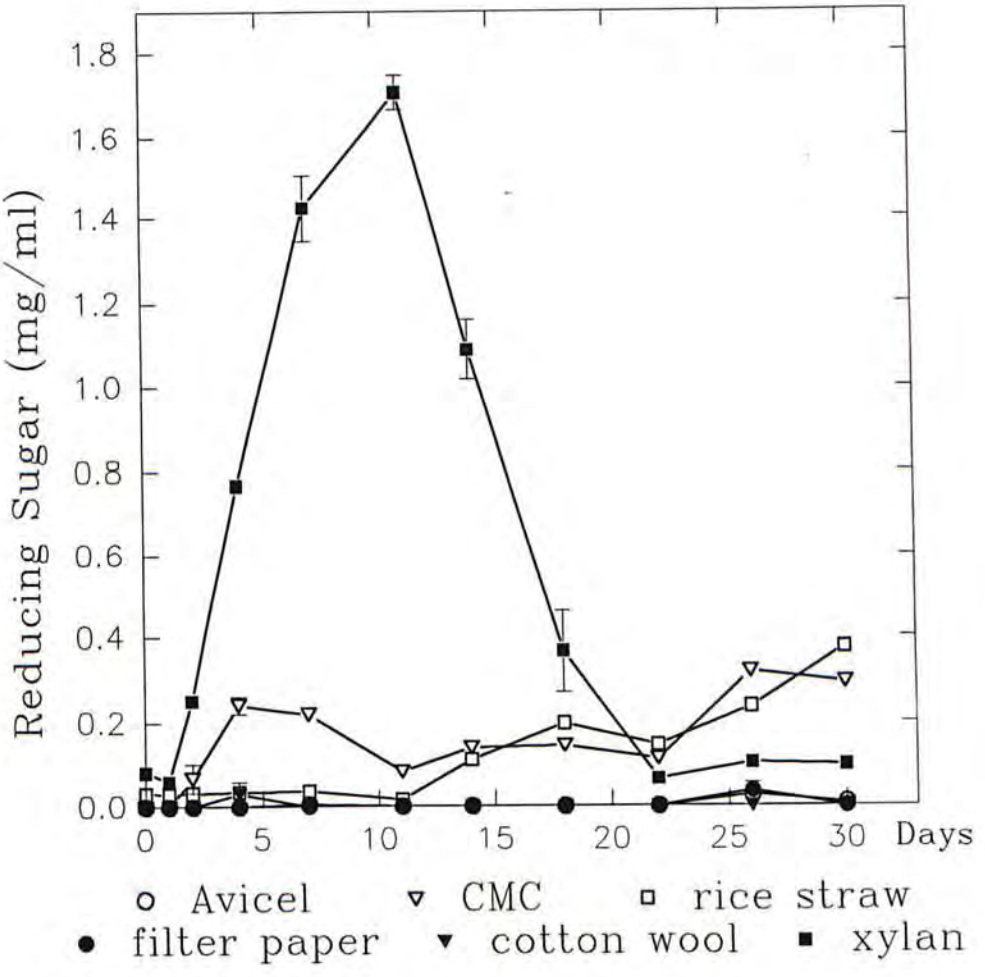

Figure 3.36 Changes in Reducing Sugar Levels in DMS Media supplemented with Different Substrates. Reducing sugar contents in Avicel, CMC, filter paper and cotton wool cultures were measured as glucose equivalents while those in birchwood xylan and rice straw cultures were measured as xylose equivalents.

3.6 Partial Purification of Different Cellulases Species Produced by *Flammulina velutipes*

3.6.1 Native-Polyacrylamide Gel Electrophoresis

Lyophilized concentrated culture filtrates of *Flammulina velutipes* grown on DMS medium containing Avicel were examined by Native-PAGE. Figure 3.37 shows a typical silver-stained slab gel obtained after electrophoresis. Based on the result of silver staining of proteins and the resolution of the Native-PAGE, approximately 13 major protein bands could be separated.

3.6.2 Activity Staining for Endoglucanases

Activity staining for endoglucanases was carried out after Native-PAGE on the lyophilized concentrated culture filtrate of *F. velutipes*. The destained regions on the agarose overlay in Figure 3.38 correspond to the locations of endoglucanases in the Native-PAGE slab gel. Although the resolution obtained using this method was not high enough to enable discrete bands to be identified, endoglucanase activity was restricted to protein bands 1 to 6. Bands 7 to 13 contained no endoglucanase activity.

3.6.3 Activity Staining for ß-Glucosidases

Activity staining for ß-glucosidases was performed after Native-PAGE on the lyophilized concentrated culture filtrate of *F. velutipes.* The yellow regions on the slab gel in Figure 3.39 represent the locations of *p*-nitrophenol resulting from ß-glucosidases activity. Due to rapid diffusion of the enzyme in the gel during incubation, no discrete bands were obtained. However, ß-glucosidase activity was restricted to

protein bands 1 and 2. No β-glucosidase activity was detected in bands 3 to 13.

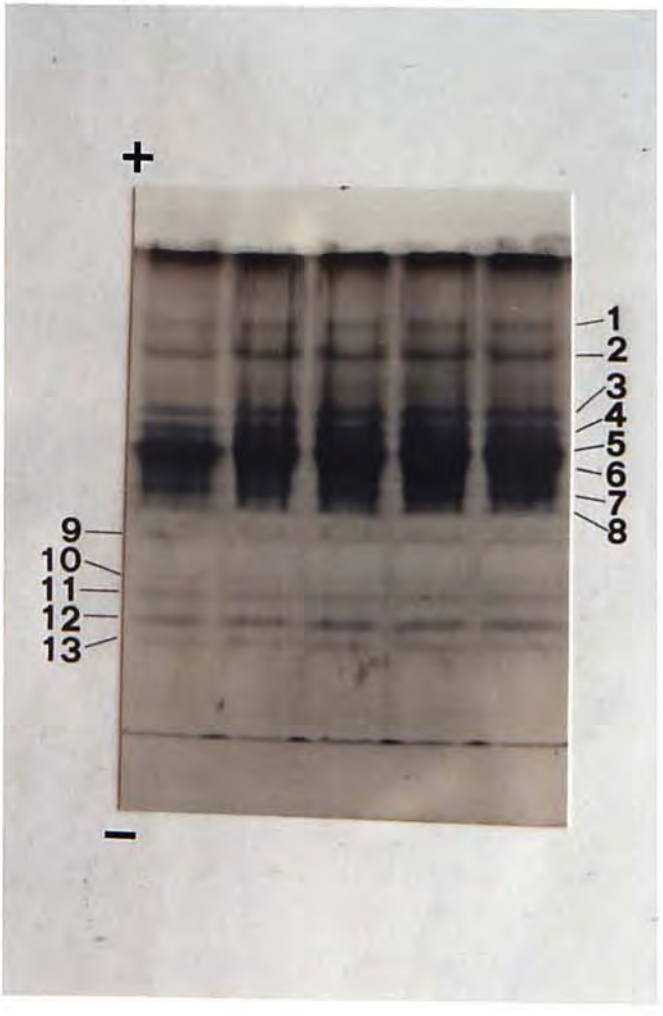

Figure 3.37 Silver-Stained Native-PAGE of Lyophilized Concentrated Culture Filtrate of *F. velutipes* Grown on DMS Medium Containing Avicel. Approximately 13 major protein bands could be separated and are referred to as band 1 to band 13 as shown.

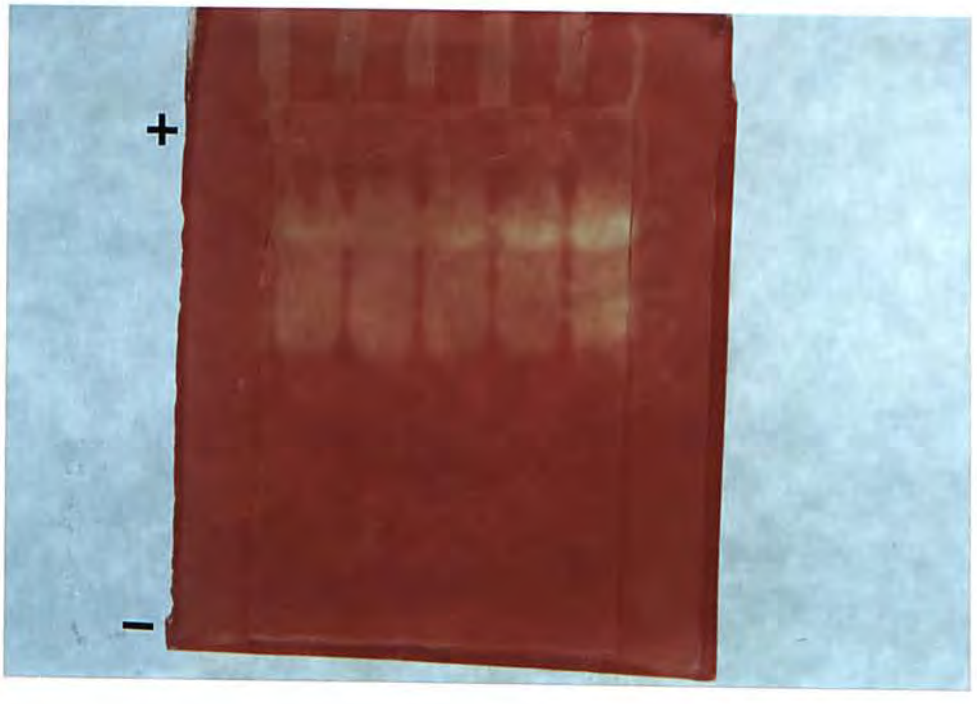

Figure 3.38 Activity Staining for Endoglucanases. Destained regions on the agarose overlay representing endoglucanase activity corresponded to protein bands 1 to 6 on the Native-PAGE slab gel (Figure 3.37).

Figure 3.39 Activity Staining for ß-Glucosidases. Yellow regions on the Native-PAGE slab gel representing ß-glucosidase activity corresponded to protein bands 1 and 2 on the Native-PAGE slab gel (Figure 3.37).

3.6.4 Assay of Cellulolytic Enzymes after Preparative Polyacrylamide Gel Electrophoresis

Thirteen horizontal strips (Strips a to m) cut out from a Preparative-PAGE slab gel were eluted with buffer and, after dialysis of the eluates against distilled water, assays for exoglucanase, endoglucanase and ß-glucosidase activities were performed. The results are presented in Table 3.2. Although the aim was to cut strips which contained only one protein band, the precise number of protein species in each strip was determined by preforming Native-PAGE on the concentrated dialysed samples. Figure 3.40 shows the silver-stained slab gel after Native-PAGE. The number of protein bands contained in each strip are given in Table 3.2. From the results of the enzyme assays, and the Native-PAGE performed on the dialysates, the number of exoglucanases, endoglucanases and ß-glucosidases present in culture filtrates of *F. velutipes* was deduced and is shown in Table 3.3. Although the results are only preliminary, at least one exoglucanase, two endoglucanases and one extracellular ß-glucosidase were detected.

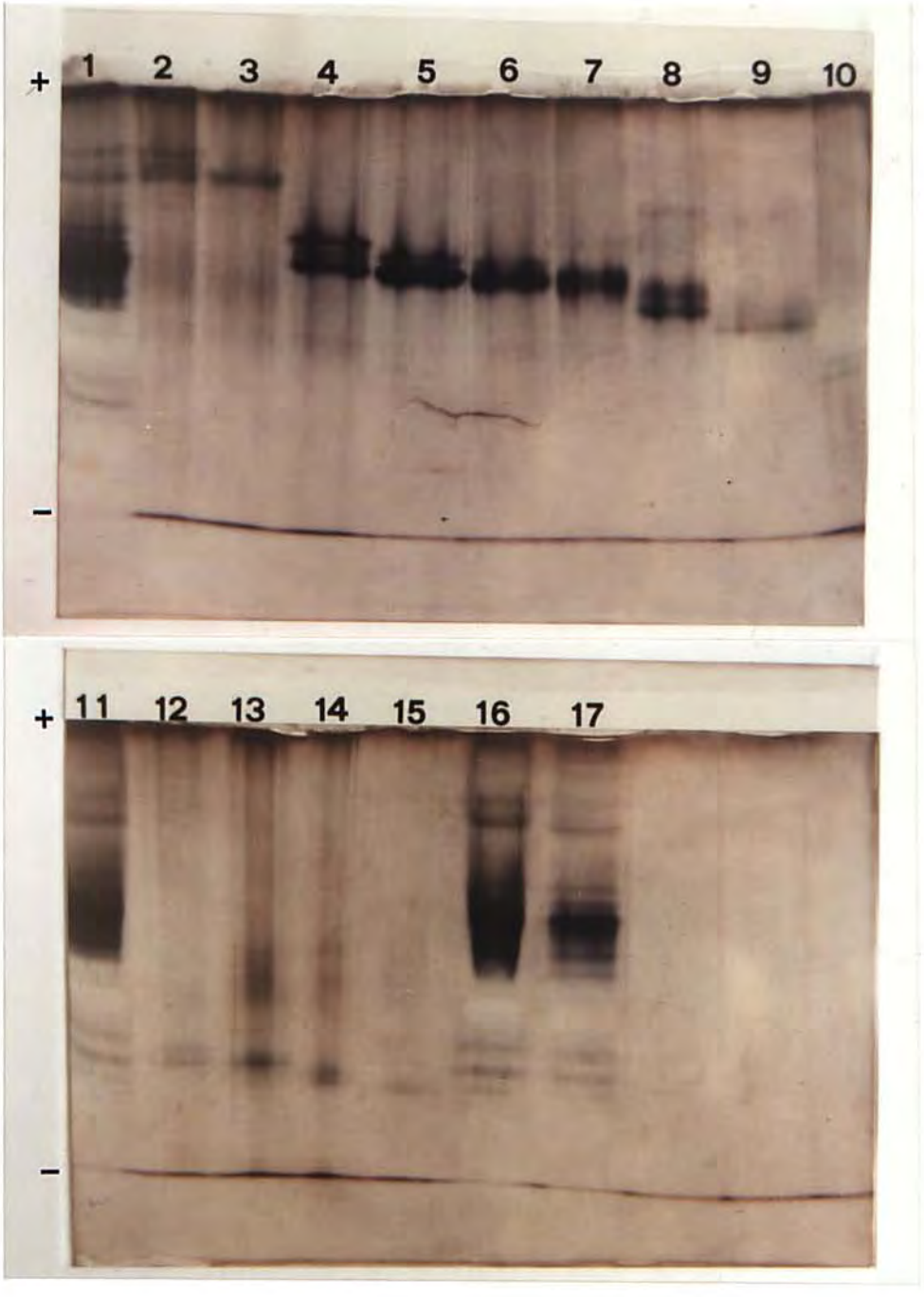

Figure 3.40 Silver-Stained Native-PAGE of Concentrated Dialysates of Gel Strips following Preparative-PAGE. Lanes 1, 11, 16 and 17: lyophilized concentrated culture filtrate. Lanes 2-10: concentrated dialysates of gel strips a-i, respectively. Lanes 12-15: concentrated dialysates of gel strips j-m, respectively.

Table 3.2 Enzyme Assays and Protein Bands as Determined by Native-PAGE on Dialysates of Gel Strips following Preparative-PAGE

Strip	Protein bands contained in strip	Exoglucanase Activity	Endoglucanase Activity	β-Glucosidase Activity
a	1,2	–	+	+
b	2	–	+	+
c	3,4,5	+	+	–
d	4,5	+	+	–
e	4,5	+	+	–
f	5,6	–	–	–
g	6,7	–	–	–
h	8	–	–	–
I	9	–	–	–
j	11,12	–	–	–
k	11,12	–	–	–
l	12,13	–	–	–
m	13	–	–	–

`+' represents detectable cellulase activity

`–' represents no detectable cellulase activity

Table 3.3 Deduced Number of Cellulase Species Produced by *F. velutipes*

Protein band in Native-PAGE	Exoglucanase Activity	Endoglucanase Activity	ß-Glucosidase Activity
1	–	?	?
2	–	+	+
3	?	?	–
4	+	+	–
5	–	–	–
6	–	–	–
7	–	–	–
8	–	–	–
9	–	–	–
10	–	–	–
11	–	–	–
12	–	–	–
13	–	–	–

`+' represents presence of cellulase activity

`–' represents absence of cellulase activity

`?' represents possible cellulase activity

CHAPTER 4
DISCUSSION

4.1 Optimal Conditions for Cellulolytic and Hemicellulolytic Enzymes of *F. velutipes*

4.1.1 Optimal Temperature for Enzymic Reaction

The results obtained in the present study show that the temperature optima for exoglucanase and endoglucanase activities of *F. velutipes* were 40°C and 50°C, respectively. Previously reported optimal temperatures for exoglucanase activities range from 40°C to 65°C (Kanda *et al.*, 1976a; Kanda *et al.*, 1976b; Beldman *et al.*, 1985; Taj-Aldeen and Alkenany, 1993). Beldman *et al.* (1985) reported that two exoglucanases from *Trichoderma viride* both had temperature optima of 50°C. Temperature optima for endoglucanases ranged from 48 to 60°C. Optimal temperatures for both endoglucanase and exoglucanase activities of *Polyporus tulipiferae* were shown to be around 50°C (Kanda *et al.*, 1976a). Another endoglucanase obtained from *Polyporus tulipiferae* was reported to have a temperature optimum of 40°C (Kanda *et al.*,1976b). However, an endoglucanase from *Aspergillus niveus* was reported to have a temperature optimum of 65°C (Taj-Aldeen and Alkenany, 1993). The results obtained in this study with *F. velutipes* are comparable to those reported in other fungi.

Conversely, the temperature optimum for the ß-glucosidase(s) of *F. velutipes* were shown to be 50°C, values which were slightly higher than those obtained elsewhere. Heupel *et al.* (1993) purified an intracellular ß-glucosidase from *Streptomyces reticuli* which had a temperature optimum at 40°C. The extracellular ß-glucosidase of the white-rot fungus *Trametes gibbosa* was reported to have an optimum

temperature for activity also at 40°C (Bhattacharjee *et al.,* 1992). However, the results obtained here are very similar to the value obtained by Beldman *et al.* (1985) who recorded the temperature optimum of *Aspergillus niveus* ß-glucosidase as 49°C.

The optimal temperature for xylanase activity of *F. velutipes* was 40°C. This value is considerably lower than reported for xylanases from other sources. Two xylanases, xylanase I and II purified from *Neocallimastix frontalis,* a rumen fungus, exhibited optimal activity at 55°C (Gomez De Segura and Fevre, 1993). A xylanase from *Thermomyces lanuginosus* had an optimal temperature for activity at 65°C (Gomes *et al.,* 1993) while xylanases from other thermophilic actinomycetes *Thermomonospora chromogena, Thermomonospora curvata, Thermomonospora fusca* and *Saccharomonospora viridis* exhibited highest enzymic activity within the temperature range from 60°C to 70°C (McCarthy *et al.,* 1985).

Both extracellular and cell-associated ß-xylosidase of *F. velutipes* are relatively thermostable and highest activities of both forms of the enzyme were recorded at 60°C. The temperature optimum of an extracellular ß-xylosidase from *Trichoderma reesei* was also 60°C (Poutanen and Puls, 1988). However, the reported optimum temperature for the intracellular ß-xylosidase from *Aspergillus niger* was greater than 75°C (Uchida *et al.,* 1992).

4.1.2 Optimal pH for Enzymic Reaction

The pH optima for exo- and endoglucanase activities of *F. velutipes* were found to be pH 7.4 and pH 7.0, respectively. Endoglucanases from other fungi were more active at acidic pH values. For example, endoglucanase from *Aspergillus niveus* (Taj-Aldeen and Alkenany, 1993) had a pH-optimum at 4.8, while the optimal pH for endoglucanase activity of *Polyporus tulipiferae* (Kanda *et al.,* 1976a)

was found to be between pH 4.0 and pH 5.0. Another endoglucanase purified from the same fungus exhibited maximum activity at pH 5 (Kanda *et al.*,1976b).

The optimum pH values for extracellular and cell-associated ß-glucosidases activities of *F. velutipes* were pH 5.4 and pH 5.8, respectively, which are similar to the optimal pH values for ß-glucosidases from other fungi. Taj-Aldeen and Alkenany (1993) recorded the pH optimum of an extracellular ß-glucosidase from *Aspergillus niveus* at pH 5.0. On the other hand, two extracellular ß-glucosidases purified from *Sporotrichum pulverulentum* were reported to have pH optima between pH 4.0 and pH 4.5 (Deshpande *et al.*, 1978). A much more acidic extracellular ß-glucosidase purified from *Trametes gibbosa* was found to have a pH optimum at pH 3.5 (Bhattacharjee *et al.*, 1992). Conversely, an intracellular ß-glucosidase from *Streptomyces reticuli* exhibited maximum activity at pH 7 (Heupel *et al.*, 1993).

The optimal pH for xylanase activity of *F. velutipes* was pH 7.0. This value was similar to optima reported for xylanases from other sources. Xylanases obtained from *Thermomonospora* spp. and *Saccharomonospora viridis* exhibited optimum activities around pH 6 and 7, respectively (McCarthy *et al.*, 1985). A xylanase of *Bacillus circulans* also had a pH optimum at pH 7 (Ratto *et al.*, 1992) while a xylanase from *Thermomyces lanuginosus* exhibited maximum activity at pH 6.5 (Gomes *et al.*, 1993). The xylanases I and II purified from *Neocallimastix frontalis* were shown to have pH optima at 5.5 and 6.0, respectively (Gomez De Segura and Fevre, 1993). In contrast, Kanda *et al.* (1976b) reported pH 4 as the optimum for xylanase activity from *Polyporus tulipiferae*.

Extracellular and cell-associated ß-xylosidase activities of *F. velutipes* were found to be maximal around pH 5.8. Similar results were obtained by Uchida *et al.* (1992) who found that an intracellular ß-xylosidase from *Aspergillus niger* had a pH optimum of pH 5.0. A more

acidic extracellular ß-xylosidase from *Trichoderma reesei* with a pH optimum at pH 4.0 was described by Poutanen and Puls (1988). In contrast, Ratto *et al.* (1992) reported an extracellular ß-xylosidase from *Bacillus circulans* with a pH optimum of pH 6.0.

It was noted that the extracellular and cell-associated ß-glucosidases shared very similar temperature-activity and pH-activity profiles. Therefore, it is possible that these two enzymes are encoded by the same gene but undergo post-translational modification as part of the secretion process.

To determine the optimal reaction time for the assay of enzymes, substrates were incubated with the enzymes for different periods of time and the products released determined. With reference to Figure 3.9, it was found that the solubilization rate of Avicel showed a decline even though a considerable amount of the substrate still remained in the reaction mixture. Since Avicel consists of more easily hydrolysable amorphous regions and less hydrolysable crystalline regions, it is speculated that the exoglucanase preferentially attacks the amorphous regions in the first ten or fifteen minutes of the reaction with a subsequent accumulation of the crystalline regions in the reaction mixture. After fifteen minutes, the number of amorphous regions may become limited and hence the solubilization rate declined.

A similar phenomenon occurred in assaying ß-xylanase activity (Figure 3.13). A lot of reducing material was released in the first five or ten minutes of reaction followed by a lower solubilization rate of the birchwood xylan. Since xylan is a heterogeneous complex substrate consisting of a ß-1,4-connected xylose backbone with highly substituted side branches, preferential attack on unsubstituted portions of the xylan backbone was expected since the presence of substituent groups would inhibit the action of endoxylanase on glycosidic linkages in the vicinity of the site of substitution (Kormelink and Voragen, 1993). After a period of time, the number of unsubstituted portions

susceptible to enzymic attack would presumably become limited and the reaction rate would then decline. For practical purposes, the reaction times for the exoglucanase and ß-xylanase assays were chosen to be 15 and 20 minutes, respectively.

4.2 Production of Cellulolytic and Hemicellulolytic Enzymes

4.2.1 Production of Cellulolytic Enzymes

Flammulina velutipes, a white-rot fungus which is generally cultivated commercially on mixture of rice-bran and sawdust produces several types of cellulolytic enzymes. With reference to Figure 3.23 and 3.24 exoglucanase, endoglucanase, extracellular and cell-associated ß-glucosidases could be detected in cultures of *F. velutipes* grown on DMS medium supplemented with crystalline cellulose, Avicel, as the major carbon source. The major enzymes involved in cellulose degradation, exo- and endoglucanase, were only detectable after 15 days of growth, which showed that these two enzymes are not constitutive. Similarly, ß-glucosidase could only be detected after 3 days of growth, showing that this enzyme was also not constitutively produced by *F. velutipes.*

Although this fungus was cellulolytic, the activities of exoglucanase and endoglucanase were lower than those reported for other cellulolytic fungi such as *Volvariella volvacea* (Cai *et al.,*1994) which has been reported to produce cellulolytic enzymes after 2 days of growth and which produced more than 0.05 IU per ml culture supernatant of exoglucanase and 0.5 IU per ml of endoglucanase when grown on Avicel. The production of low levels of cellulases indicated that pure cellulose substrates are unsuitable for the growth of *F. velutipes*.

Most of the ß-glucosidase activity in *F. velutipes* was found to be located extracellularly. ß-Glucosidase often remains cell-bound in bacteria. For example, *Streptomyces hygroscopicus* (Spear *et al.*,1993) had only 10% of the total enzyme activity present in the culture filtrate while extracellular ß-glucosidase activity was almost undetectable in cultures of *Thermoactinomyces* spp. (Hagerdal *et al.*,1979). In contrast, Taj-Aldeen and Alkenany (1993) reported that 74.4% of the total ß-glucosidase activity in *Aspergillus niveus* cultures was located extracellularly. Similarly, Taj-Aldeen (1993) reported that 81% of the total ß-glucosidase activity in *Trichoderma reesei* cultures was located extracellularly. According to Spear *et al.* (1993), while glucose was the major end-product resulting from the action of extracellular fungal cellulases on cellulose, the action of bacterial cellulases on cellulose results in the production of the disaccharide cellobiose. The virtual absence of extracellular ß-glucosidase activity in bacterial cultures suggests a mechanism for assimilation of cellulose which may involve the initial extracellular conversion to cellobiose, followed by a cell-associated conversion of cellobiose to glucose. In the case of *F. velutipes*, since more extracellular ß-glucosidase than cell-associated ß-glucosidase was found in the fungal cultures, it can be speculated that the major end-product resulting from the action of the fungus's cellulases on cellulose was also glucose. Hence, the mushroom does not need a high cell-associated ß-glucosidase activity for intracellular cellobiose degradation.

4.2.2 Production of Hemicellulolytic Enzymes

The results of the present study reveal that *Flammulina velutipes* is a better producer of xylanolytic enzymes than cellulolytic enzymes. The fungus produced high xylanase activity when grown in a medium supplemented with birchwood xylan as the major carbon source. This

compares favourably to another edible mushroom, *Volvariella volvacea,* which produced no detectable xylanase and only low amounts of xylanase and ß-xylosidase when the fungus was grown on birchwood xylan and rice straw medium, respectively (Cai *et al.,* 1994). *F. velutipes* appears more adaptable to growth on substrates with a higher hemicellulose to cellulose ratio.

A study by Leatham (1985) on another commercially important white-rot mushroom, *Lentinula edodes,* showed that the fungus is only moderately cellulolytic and hence grows with some difficulty on native cellulose as a sole carbon source. In contrast to cellulases, hemicellulases were extracted in high titers from cultures of *L. edodes.* The data in the present study suggest that, in this respect, *F. velutipes* is very similar to *L. edodes* in being a poor cellulolytic but highly hemicellulolytic mushroom.

Since *F. velutipes* is highly hemicellulolytic, accumulation of the products of xylan degradation in the culture medium is to be expected. It is well established that xylanases and xylosidases are subject to end-product inhibition (Ball and McCarthy, 1989; Bachmann and McCarthy, 1991; Rothlisberger *et al.,* 1992; Uchida *et al.,* 1992). According to the present results, ß-xylosidase activity detected in xylan-supplemented cultures began to stabilize on day 11 when reducing sugar levels peaked. This suggests that reducing sugar in the culture medium may have an inhibitory effect on xylanase production by the fungus. However, the effect of end-product inhibition on extracellular ß-xylosidase production was not as evident as that on ß-xylanase.

4.3 Enzyme Induction Patterns

Certain gene products are essential components of living cells. Genes that specify products of this type are continuously being

expressed in most cells. Such genes are said to be expressed constitutively and are referred to as constitutive genes.

Other gene products are needed for cell growth only under certain environmental conditions. Constitutive synthesis of such gene products would clearly be wasteful, using energy that could otherwise be utilized for more rapid growth and reproduction under the existing environmental conditions. Organisms therefore evolved a regulatory mechanism to control the expression of genes in response to different environmental conditions. The process, by which the expression of genes is turned on in response to a substance in the environment, is called induction. Genes whose expression are so regulated are called inducible genes; their products, if enzymes, are called inducible enzymes. The substances or molecules responsible for induction are known as inducers. Enzymes that are involved in catabolic (degradative) pathways, are characteristically inducible. Induction occurs at the level of transcription. It alters the rate of synthesis of enzymes, not the activity of existing enzyme molecules.

In another regulatory mechanism by which the synthesis of gene products is turned off, the gene is said to be repressed. When its expression is turned on, a gene of this type is said to be derepressed. Enzymes that are components of anabolic (biosynthetic) pathways are frequently subject to repression.

Cellulases and hemicellulases are inducible enzymes (see Section 1.4.2 and 1.4.4). Results presented here suggest that rice straw is the best substrate for cellulases and hemicellulases production by *F. velutipes*. Activities of exoglucanase, extracellular ß-glucosidase, ß-xylanase and extracellular ß-xylosidase in rice straw-supplemented media were the highest among enzyme levels observed in the other induction media tested. Indeed, complex substrates such as bagasse, wheat bran (Teunissen *et al.*, 1992), cotton-wheat straw (Masaphy and Levanon, 1992), wheat straw (Teunissen *et al.*, 1992; Maheswari *et al.*,

1993) and rice husks (Kuhad and Singh, 1993) have previously been shown to induce cellulase and/or xylanase production.

The present results are actually consistent with those obtained elsewhere where complex substrates or less readily available substrates were generally better enzyme inducers than soluble or more readily-available substrates. Bailey *et al.* (1993) demonstrated that the less readily available, insoluble beech xylan substrate effected induction of high xylanase activity in *Trichoderma reesei* in the absence of any other inducers. The present results are also in accordance with similar findings obtained by Biely *et al.* (1980) who reported that enzyme induction by xylan continued for a longer time than when induction was achieved using more available xylose oligosaccharides. Therefore, it seems that low substrate availability may be an important factor for efficient enzyme production.

Hemicelluloses from different sources, i.e. grasses, cereals, softwoods, and hardwoods, differ in their composition. For example, xylan from grasses and cereals have various amounts of arabinofuranosyl substituents and glucopyranosyl uronic acid substituents and may also be acetylated to a low degree (Kormelink and Voragen, 1993). The complex nature of hemicellulose in rice straw may induce the production of more branch-cleaving enzymes than pure birchwood xylan. These branch-cleaving enzymes will in turn enable more effective attack by ß-xylanase on the xylan backbone. It is possible that the hemicellulose substituents, apart from inducing branch-cleaving enzymes, also induce other enzymes of the hemicellulolytic enzyme system such as ß-xylanase. So, ß-xylanase activity in rice straw medium would be higher than levels observed in xylan medium.

Alternatively, since rice straw is a chemically complex substrate, it is reasonable to speculate that some unknown component(s) in the rice straw serve as the true inducer(s) of the cellulases and hemicellulases.

While complex substrates appear to be better enzyme inducers than pure substrates, different pure celluloses also have different potentials for cellulases induction. How is this potential related to the structure of a cellulose substrate?

Celluloses chosen in the present study included cotton wool, Avicel, CMC and filter paper which could be considered as relatively pure cellulose. Structurally, cotton wool most closely resembles native cotton fibre which is an archetypal crystalline cellulose with a chain length of 10,000 to 15,000 glucose residues (Wood, 1991). It was speculated that such a high degree of polymerization, insoluble nature, and a relatively low surface area available to enzymic attack would be the major reasons why cotton wool was found to be a relatively poor substrate for cellulolytic enzymes production in the present study.

Avicel is a type of microcrystalline cellulose. It is a highly hydrogen bond-ordered cellulose. Compared with cotton fibre, Avicel has a relatively shorter cellulose chain length of about 200 glucose residues (Wood, 1991). Hence, there exist more reducing end groups per unit weight compared to other forms of crystalline cellulose. Avicel is commonly employed as the substrate for the assay of endwise acting enzymes such as exoglucanases. On the assumption that accessibility and the crystallinity of cellulosic substrates are the major factors affecting hydrolysis, an effective pretreatment of native cellulose will make the internal area of the porous cellulose fibers more accessible to the cellulase system. When compared to cotton wool, Avicel seems to have a relatively larger surface area available to enzymes and a lower degree of polymerization. These factors may account for the production of higher levels of cellulolytic enzymes in Avicel-supplemented cultures.

Carboxymethylcellulose, CMC, contains a non-uniform distribution of substituted residues. This compound loses its initial ordered structure and becomes susceptible to attack by the cellulase system. However, cellulase systems can in fact break glycosidic linkages only where there

are two or more contiguous unsubstituted anhydroglucose units. Therefore, a lower glucose yield will be expected in the degradation of highly substituted CMC (Focher *et al.*, 1991). Since CMC is soluble, it is expected that it will be more effective than other forms of cellulose as an inducer of cellulolytic enzymes. However, although CMC-supplemented medium seemed to be a good inducer of exoglucanase production in the present study, it did not have any marked effect on the induction of endoglucanase and extracellular ß-glucosidase. Determination of the reducing sugar content (Figure 3.36) in the CMC medium revealed that the degradation of CMC was rapid and efficient even though only small amounts of cellulases were detectable during the first few days of cultivation. A considerable increase in the reducing sugar content was observed in the induction medium while the levels of enzymes remained quite low. Therefore, the possibility of end-product inhibition of enzyme activities cannot be ruled out in CMC medium.

Filter paper is also modified cellulose. Due to the physical and chemical treatment in the process of making filter paper from plant materials, a certain degree of structural change in the highly crystalline property of native cellulose is to be expected. A transition from crystalline to amorphous state will be the common case. Therefore, it is expected that filter paper would have more amorphous regions than cotton wool. On the basis of the assumption that a preferential attack occurs on the amorphous fractions of cellulose over the crystalline fractions, higher enzymes activities should be induced in filter paper-containing medium than in cotton wool-containing medium. This hypothesis was supported by the data of Chahal *et al.* (1992) who demonstrated that a drop in the cellulase production profile was evident during cellulose degradation by *Trichoderma reesei.* These authors suggested that this phenomenon was partly due to a shift from amorphous to crystalline cellulose. The present results also revealed that filter paper-supplemented medium was more effective than cotton

wool-supplemented medium in the induction of exoglucanase and was even more effective than cotton wool-containing culture in the induction of endoglucanase and extracellular ß-glucosidase. Furthermore, the slow release of reducing sugars in filter paper medium also helped to relieve the end-product inhibition on enzymic activity.

In conclusion, media containing complex substrates are far more effective than those supplemented with pure cellulose or xylan in the induction of cellulases and hemicellulases. This might be explained by, firstly, the reduced accessibility of the substrate, rice straw. Secondly, the heterogeneous, complex structure of rice straw hemicellulose might be more effective than purified xylan in the induction of an array of hemicellulolytic or xylanolytic enzymes. Finally, some unknown component(s) in the rice straw or natural substrates might serve as the true inducer(s) of cellulolytic or hemicellulolytic enzymes. On the other hand, the induction potential of pure cellulosic substrates seems to be more closely related to the structural features of the cellulosic materials. Several factors such as the availability of surface area, particle size, crystallinity and degree of polymerization of cellulose are possible explanations for the differences in the enzymic activities induced. These possible explanations are also currently under extensive investigations and challenged by several researchers (Dermoun and Belaich, 1988: Chahal *et al.,* 1992; Nidetzky and Steiner, 1993).

4.4 Partial Purification of Different Cellulase Species Produced by *Flammulina velutipes*

Purification of cellulose-degrading enzymes are usually focused on high cellulase-producing species. Typical cellulolytic fungi extensively studied included *Trichoderma* spp., *Phanerochaete chrysosporium* and *Penicillium* spp. Wood and McCrae (1972) isolated the C_1 component of the cellulolytic enzyme system of *Trichoderma koningii* by

chromatography on DEAE-Sephadex. This C_1 component was later identified as a ß-1,4-glucancellobiosylhydrolase (exoglucanase). In 1978, a cellobiase, a CM-cellulase, a C_2 component and a cellobiohydrolase from the culture filtrates of the same fungus were isolated and purified by affinity chromatography (Halliwell and Griffin, 1978). In the same year, Wood and McCrae (1978) further separated and purified four endoglucanase components of the cellulase complex of *T. koningii* by gel filtration, ion-exchange chromatography and isoelectric focusing.

In another species of *Trichoderma, Trichoderma reesei,* two cellobiohydrolases, CBHI and CBHII, were efficiently separated by chromatography on DEAE-Sepharose and isoelectric focusing (Fagerstam and Pettersson, 1980). In 1984, Van Tilbeurgh *et al.* separated two cellobiohydrolases, CBHI and CBHII, and an endoglucanase from the same fungus by using a new affinity chromatography method. A study by Beldman *et al.* (1985) on *Trichoderma viride* also showed that six endoglucanases (Endo I; II; III; IV; V; VI), three exoglucanases (Exo I; II; III) and a ß-glucosidase (ß-gluc I) could be isolated from a commercial cellulase preparation derived from the fungus by using gel filtration, anion exchange on DEAE-Bio-Gel A, cation exchange on SE-Sephadex and affinity chromatography on crystalline cellulose.

Apart from *Trichoderma,* the cellulase system of *Penicillium pinophilum* is also well-studied. Two immunologically unrelated cellobiohydrolases (I and II) (Wood and McCrae, 1986; Wood *et al.,* 1989) and five major endoglucanases (Wood *et al.,* 1989) were isolated from the fungus. Based on the experimental results on the synergistic activities of the isolated isoenzymes, Wood and McCrae (1986) further proposed a hypothesis to explain the phenomenon of exo-exo synergism. They suggested that the cellobiohydrolases may be two stereospecific enzymes concerned with the hydrolysis of the two

different configurations of non-reducing end groups that would exist in cellulose.

The cellulolytic enzyme system of a white-rot fungus, *Phanerochaete chrysosporium,* has also been studied extensively. Deshpande *et al.* (1978) purified and partially characterized five ß-glucosidases from *Phanerochaete chrysosporium.* Three exoglucanases (CBHI, CBH50 and CBH62) were also isolated from the culture filtrate of this fungus by chromatography on DEAE-Sepharose (Uzcategui *et al.,* 1991).

Although information and studies on the cellulolytic enzymes of fungi are well-documented and much effort has been made in this area of research, reports on the purification and characterization of the cellulases of commercially important edible mushroom are rare and limited. In 1990, Mishra and Leatham reported an initial purification and identification by anion exchange chromatography of the range of enzymes present in a crude culture filtrate of *Lentinula edodes* (Shiitake mushroom) cultivated on a solid lignocellulosic substrate. The enzymes included cellulases, hemicellulases, fungal cell wall-degrading enzymes, oxidative enzymes (ligninases), acid phosphatases, and acid proteinases. Although these enzymes were not purified to homogeneity, their study revealed that culture filtrates of *L. edodes* contained at least two exoglucanases, four endoglucanases and one ß-glucosidases. Although not likely to be an economically-viable method for the commercial production of these enzymes, recovery offers the opportunity to produce enzyme quantities sufficient for characterization and the development of potential industrial applications.

The number of different cellulase species produced by *F. velutipes* in the present study was deduced from results of activity staining on Native-PAGE slab gel and enzyme assays on the proteins eluted from the strips cut out from the slab gel after Preparative-PAGE. Activity staining for endoglucanase activity is based on the fact that

Congo red binds strongly to polysaccharides containing adjacent ß-1,4-linked glucopyranosyl residues. Hence, gels after electrophoresis could be overlaid by thin sheets of agar containing carboxymethylcellulose. After incubation and staining of the agar replica with Congo red, the locations corresponding to the endocellulases were revealed by their lack of colour. Although the resolution of this method was not high enough to enable discrete bands to be discerned, especially in using crude samples, endoglucanase activity was found to be restricted to protein bands 1 to 6. Qualitative detection of ß-glucosidase activity can also be performed by activity staining on Native-PAGE slab gel. The method employed is relatively simpler and more direct than that for endoglucanase. The method makes use of the fact that the activity of ß-glucosidase on PNPG releases PNP which is bright yellow in colour under alkaline conditions. Hence, yellow bands on the gel after electrophoresis indicate the presence of enzymic activity. Since the colour of the product is yellow, accumulation of significant amounts of PNP is required to obtain a deep yellow band giving an acceptable contrast between the band and the background. Therefore, a longer time of incubation would be required. The problem of long-time incubation is the unavoidable diffusion of enzyme and product in the gel and the result is that discrete bands are difficult to obtain. In spite of this, the results of activity staining indicated ß-glucosidase activity was restricted to protein bands 1 and 2.

Results based on activity staining, gel-cutting from Preparative-PAGE slab gel and subsequent enzyme assays suggested that *Flammulina velutipes* produced at least one exoglucanase, two endoglucanases and one extracellular ß-glucosidase in the DMS induction medium containing Avicel. Due to the poor resolution of the Native-PAGE and the fact that protein bands were too close to be separated completely by cutting gel strips from the Preparative-PAGE, it is not easy to tell whether some of the protein bands contain cellulases

activities (Table 3.3). Nevertheless, it is interesting to find that protein band 2 contained both endoglucanase and ß-glucosidase activities while protein band 4 contained both exoglucanase and endoglucanase activities. The phenomenon of broad substrate- or multi-specificity of enzymes is common and has frequently been reported by researchers. In 1978, Wood and McCrae isolated four endoglucanases from *Trichoderma koningii* which could hydrolyse CMC, H_3PO_4-swollen cellulose, cellotetraose and cellopentaose, but differed in the rate and mode of attack. Beldman *et al.* (1985) also found a ß-glucosidase from *Trichoderma viride* active on CMC, crystalline cellulose, xylan, *p*-nitrophenyl-ß-glucose and *p*-nitrophenyl-ß-xylose. A purified endocellulase of the Avicelase type from *Polyporus tulipiferae* was also able to attack a series of cellooligosaccharides, ß-cellobioside, CMC, and insoluble cellulosic substrates (Kanda *et al.*, 1976a). Therefore, it is perhaps not surprising to find that *F. velutipes* also produces enzymes with multispecificity.

On the other hand, it is also possible that the resolution of the Native-PAGE was not high enough so that two or more proteins appeared as a single band on the gel. Hence, the broad-substrate specificity of a `single' protein may actually be due to the activities of two or more proteins in the `single' band.

As the present results suggested (Table 3.3), *F. velutipes* appears to produce more than one kind of exoglucanase and ß-glucosidase and more than two kinds of endoglucanases in the culture filtrate. Very often, the mechanism of cellulase action is discussed in terms of the type of enzyme rather than in terms of the number of enzymes required. In fact, as discussed before, it is common that multiple forms of cellulases are produced by a single fungus. The reason for the multiplicity of enzyme forms has, in the past, given rise to much conjecture. Wood and McCrae (1986) have argued that, for stereochemical reasons, at least two types of endoglucanases and two

exoglucanases are necessary for hydrolysis of cellulose. However, it is still in question whether each of the individual forms is genetically determined, or arises from differential modification by proteolysis or glycosylation etc. inside or outside the cell.

4.5 Conclusion

Flammulina velutipes is an economically important edible mushroom. Cultivation of this fungus is usually carried out on sawdust and rice bran mixtures which has been recognized as an ideal method for commercial exploitation. In spite of its economic importance, little is known about the ability of the fungus to degrade the cellulose, hemicellulose and lignin components of the growth substrates. The present investigation provided some insights into the capability of the fungus to utilize the major polymeric components of a particular substrate as a source of nutrition for growth and fruiting.

Preliminary results on the investigation on the lignocellulolytic enzyme profile of *F. velutipes* grown on DMS medium supplemented with Avicel or birchwood xylan revealed that this fungus could produce measurable amounts of exoglucanase (Avicelase), endoglucanase (carboxymethylcellulase), extracellular and cell-associated ß-glucosidase, ß-xylanase, extracellular and cell-associated ß-xylosidase. The temperature and pH optima for enzymic activity were determined as 40, 50, 50, 50, 40, 60 and 60°C and pH 7.4, 7.0, 5.4, 5.8, 7.0, 5.8 and 5.8, respectively.

Time course experiments conducted to obtain the profile of lignocellulolytic enzyme production by *F. velutipes* grown on Avicel- or birchwood xylan-supplemented DMS medium showed that the major enzymes involved in cellulose degradation, exo- and endoglucanase, only became detectable after 15 days of growth implying that this fungus is only moderately cellulolytic. The fact that the level of

extracellular ß-glucosidase was found to be much higher that that of cell-associated one, is in accordance with other fungal cellulolytic enzyme systems. The present data also suggested that this fungus was a better producer of xylanolytic than cellulolytic enzymes. Hence, *F. velutipes* was clearly more suitable for cultivation on substrates with a higher hemicellulose to cellulose ratio.

Enzyme induction pattern obtained by transferring PDB-grown fungal biomass to DMS media containing different substrates (Avicel, filter paper, carboxymethylcellulose, cotton wool, rice straw and birchwood xylan) for enzyme production revealed that rice straw was the best substrate for the production of both cellulolytic and xylanolytic enzymes. A possible explanation accounting for the high inductive potential of rice straw might be the low accessibility of the substrate to enzymic attack. Furthermore, the heterogeneous nature of rice straw might be more effective in the induction of enzymes. Another possibility is that some unknown component(s) in the rice straw might serve as the true inducer(s) of cellulolytic and/or hemicellulolytic enzymes.

Results on the partial purification of cellulase species of *F. velutipes* suggested that this fungus produced at least one exoglucanase, two endoglucanases and one extracellular ß-glucosidase when grown in DMS medium supplemented with Avicel. Some protein bands on the Native-PAGE were found to have multispecificity. It is still uncertain whether this property was intrinsic to the enzymes themselves or just an artifact resulted from poor resolution on the Native-PAGE. Nevertheless, cutting gel strips from Preparative-PAGE followed by eluting the proteins from the gel has provided a quick and simple method for the partial purification of different cellulase species produced by *F. velutipes*. To obtain a more reliable result, it is suggested that the conditions of running gel electrophoresis should be modified so that the protein bands can be well-separated in the electrophoretic run thereby enabling only one protein to be cut out in a

single gel strip. On the other hand, this method seems to be more useful when used in combination with other enzyme purification techniques such as ammonium sulphate precipitation, gel filtration, or ion-exchange chromatography. It is often very time-consuming to try out many methods to separate two or more proteins with similar physical and chemical properties. Therefore, enzymes from crude samples, purified to almost homogeneity by the common enzyme purification techniques, can be more easily and quickly brought to purity by simply applying the method of cutting gel from Preparative-PAGE.

4.6 Further Studies

The present results suggested that *Flammulina velutipes* was highly xylanolytic but only moderately cellulolytic. Further studies on the mushroom may therefore focus on the production, purification and characterization of the different enzyme species of the hemicellulolytic enzyme systems. Enzyme production profiles of *F. velutipes* grown on sawdust-rice bran mixtures, the natural substrate in the commercial cultivation of the mushroom, may also be determined and compared to those obtained in cultures grown on purified lignocellulosic components. The different cellulolytic and hemicellulolytic enzyme species induced in the natural growth substrate may also be investigated and compared to those obtained in the present study.

List of References

Aschan, K. and Norkrans, B. 1953. A Study in the Cellulolytic Variation for Wildtypes and Nutrients of *Collybia velutipes.* I. *Physiologia Plantarum* **6**:565-583.

Ayers, A. R., Ayers, S. B. and Eriksson, K. E. 1978. Cellobiose Oxidase, Purification and Partial Characterization of a Hemo-protein from *Sporotrichum pulverulentum. European Journal of Biochemistry* **90**:171-181.

Bachmann, S. L. and McCarthy, A. J. 1991. Purification and Cooperative Activity of Enzymes Constituting the Xylan-Degrading System of *Thermomonospora fusca. Applied and Environmental Microbiology* **57**:2121-2130.

Bailey, M. J. and Viikari, L. 1993. Production of Xylanases by *Aspergillus fumigatus* and *Aspergillus oryzae* on Xylan-based Media. *World Journal of Microbiology and Biotechnology* **9**:80-84.

Bailey, M. J., Buchert, J. and Viikari, J. 1993. Effect of pH on Production of Xylanase by *Trichoderma reesei* on Xylan- and Cellulose-based media. *Applied Microbiology and Biotechnology* **40**: 224-229.

Ball, A. S. and McCarthy, A. J. 1989. Production and Properties of Xylanase from Actinomycetes. *Journal of Applied Bacteriology* **66**:439-444.

Beldman, G., Searle-van Leeuwen, M. F., Rombouts, F. M. and Voragen, F. G. J. 1985. The Cellulase of *Trichoderma viride. European Journal of Biochemistry* **146**:301-308.

Beldman, G., Voragen, A. G. J., Rombouts, F. M. and Pilnik, W. 1986. Communications to the Editor: Synergism in Cellulose Hydrolysis by Endoglucanases and Exoglucanases Purified from *Trichoderma viride. Biotechnology and Bioengineering* **31**:173-178.

Bhattacharjee, B., Roy, A. and Majumder, A. L. 1992. *ß*-Glucosidase of a White-rot Fungus *Trametes gibbosa*. *Biochemistry International* **28**:783-793.

Biely, P., Kratky, Z., Vrsanska, M. and Urmanicova, D. 1980. Induction and Inducers of Endo-1,4-ß-xylanase in the yeast *Cryptococcus albidus*. *European Journal of Biochemistry* **108**:323-329.

Borneman, W. S., Akin, D. E. and Ljungdahl, L. G. 1989. Fermentation Products and Plant Cell-Wall-Degrading Enzymes Produced by Monocentric and Polycentric Anaerobic Ruminal Fungi. *Applied and Environmental Microbiology* **55**:1066-1073.

Buchert, J., Ranua, M., Kantelinen, A. and Viikari, L. 1992. The Role of Two *Trichoderma reesei* Xylanases in the Bleaching of Pine Kraft Pulp. *Applied Microbiology and Biotechnology* **37**:825-829.

Buswell, J. A., Cai, Y. J. and Chang, S. T. 1993. Fungal and Substrate-Associated Factors Affecting the Ability of Individual Mushroom Species to Utilize Different Lignocellulosic Growth Substrates. Abstract S-5, *First International Conference on Mushroom Biology and Mushroom Products,* Hong Kong.

Cai, Y. J., Buswell, J. A. and Chang, S. T. 1994. Production of Cellulases and Hemicellulases by the Straw Mushroom, *Volvariella volvacea*. *Mycological Research* **98** (in Press).

Calza, R. E., Irwin, D. C. and Wilson, D. B. 1985. Purification and Characterization of Two ß-1,4-Endoglucanases from *Thermomonospora fusca*. *Biochemistry* **24**:7797-7804.

Cavicchioli, R. and Watson, K. 1991. Molecular Cloning, Expression, and Characterization of Endoglucanase Genes from *Fibrobacter succinogenes* AR1. *Applied and Environmental Microbiology* **57**:359-365.

Chahal, P. S., Chahal, D. S. and Andre, G. 1992. Cellulase Production Profile of *Trichoderma reesei* on Different Cellulosic Substrates at Various pH levels. *Journal of Fermentation and Bioengineering* **74**:126-128.

Chang, S. C. and Steinkraus, K. H. 1982. Lignocellulolytic Enzymes Produced by *Volvariella volvacea*, the Edible Straw Mushroom. *Applied and Environmental Microbiology* **43**:440-446.

Chang, S. T. 1991. Specialty Mushrooms in Asia with Emphasis on *Lentinus, Flammulina* and *Volvariella*. *Mushroom News* **39**:11-17.

Chang, S. T. and Miles, P. G. 1989. *Edible Mushrooms and their Cultivation*. CRC Press Inc., Boca Raton, USA.

Chen, S. and Wayman, M. 1992. Novel Inducers Derived from Starch for Cellulase Production by *Trichoderma reesei. Process Biochemistry* **27**:327-334.

Christov, L. P. and Prior, B. A. 1993. Xylan Removal From Dissolving Pulp Using Enzymes of *Aureobasidium pullulans. Biotechnology Letter* **15**:1269-1274.

Copa-Patino, J. L., Kim, Y. G. and Broda, P. 1993. Production and Initial Characterization of the Xylan-degrading System of *Phanerochaete chrysosporium. Applied Microbiology and Biotechnology* **40**:69-76.

Coughlan, M. P. 1985. The Properties of Fungal and Bacterial Cellulases With Comment on their Production and Application. *Biotechnology and Genetic Engineering Reviews* **3**:39-109.

Dekker, R. F. H. 1985. Biodegration of Hemicelluloses. In *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T., ed.), Academic Press, New York.

Dermoun, Z. and Belaich, K. P. 1988. Crystalline Index Change in Cellulose During Aerobic and Anaerobic *Cellulomonas uda* Growth. *Applied Microbiology and Biotechnology* **27**:399-404.

Deshpande, V., Eriksson, K. E. and Pettersson, B. 1978. Production, Purification and Partial Characterization of 1,4-ß-Glucosidase Enzymes from *Sporotrichum pulverulentum. European Journal of Biochemistry* **90**:191-198.

Eriksson, K. E. and Hamp, S. G. 1978. Regulation of Endo-1,4-ß-glucosidase Production in *Sporotrichum pulverulentum. European Journal of Biochemistry* **90**:183-190.

Eriksson, K. E. and Wood, T. M. 1985. Biodegradation of Cellulose. In *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T., ed.), Academic Press, New York. pp.469.

Eriksson, K. E. L., Blanchette, R. A. and Ander, P. 1990. *Microbial and Enzymatic Degradation of Wood and Wood Components.* Springer-Verlag, Germany.

Eriksson, K. E. L., Habu, N. and Samejima, M. 1993. Recent Advances in Fungal Cellobiose Oxidoreductases. *Enzyme and Microbial Technology* **15**:1002-1008.

Fagerstam, L. G. and Pettersson, L. G. 1980. The 1,4-ß-Glucan Cellobiohydrolases of *Trichoderma reesei* QM9414 - A New Type of Cellulolytic Synergism. *FEBS Letters* **119**:97-100.

Focher, B., Marzetti, A., Beltrame, P. L. and Carniti, P. 1991. Structural Features of Cellulose and Cellulose Derivatives, and their Effects on Enzymatic Hydrolysis. In *Biosynthesis and Biodegradation of Cellulose* (Haigler, C. H. and Weimer, P. J., eds.), Marcel Dekker, Inc., New York. pp.305.

Gamerith, G., Groicher, R., Zeilinger, S., Herzog, P. and Kubicek, C. P. 1992. Cellulase-poor Xylanases Produced by *Trichoderma reesei* RUT C-30 on Hemicellulose Substrates. *Applied Microbiology and Biotechnology* **38**:315-322.

Gelhaye, E., Petitdemange, H. and Gay, R. 1993. Adhesion and Growth Rate of *Clostridium cellulolyticum* ATCC 35319 on Crystalline Cellulose. *Journal of Bacteriology* **175**:3452-3458.

Ginterova, A. 1989. *Pleurotus* in Modern Agricultural Production. In *Proceedings of the Tewlfth International Congress on the Science and Cultivation of Edible Fungi* held in Braunschweig-Germany (FRG) 1987. pp.99-107.

Ginterova, A. and Lazarova, A. 1987. Degradation Dynamics of Lignocellulose Materials by Wood-rotting *Pleurotus* Fungi. *Folia Microbiologica* **32**:434-437.

Ginterova, A. and Lazarova, A. 1989. Energy Transformation of Lignocellulosics into Fruit Bodies of the Wood-rotting Fungus *Pleurotus ostreatus. Folia microbiologica* **34**:141-145.

Ginterova, A., Hrabovcova, J., Manzur, M., Gutierrez, I. and Lois, J. 1992. Cultivation of Fungi on Sugar Cane Biomass. *Folia Microbiologica* **37**:60-65.

Gokhale, D. V., Patil, D. G. and Bastawde, K. B. 1992. Protection of *Aspergillus niger* Cellulases by Urea During Growth on Glucose or Glucerol Supplemented Media. *Applied Biochemistry and Biotechnology* **37**:11-17.

Gomes, J., Gomes, I., Kreiner, W., Esterbauer, H., Sinner, M. and Steiner, W. 1993. Production of High Level of Cellulase-free and Thermostable Xylanase by a Wild Strain of *Thermomyces lanuginosus* Using Beechwood Xylan. *Journal of Biotechnology* **30**:283-297.

Gomez De Segura, B. and Fevre, M. 1993. Purification and Characterization of Two 1,4-ß-Xylan Endohydrolases from the Rumen Fungus *Neocallimastix frontalis. Applied and Environmental Microbiology* **59**:3654-3660.

Hagerdal, B., Harris, H. and Pye, E. K. 1979. Association of ß-Glucosidase with Intact Cells of *Thermoactinomyces. Biotechnology and Bioengineering* **21**:345-355.

Halliwell, G. and Griffin, M. 1978. Affinity Chromatography of the Cellulase System of *Trichoderma koningii. Biochemical Journal* **169**:713-715.

He, L., Bickerstaff, G. F., Paterson, A. and Buswell, J. A. 1993. Purification and Partial Characterization of Two Xylanases that Differ in Hydrolysis of Soluble and Insoluble Xylan Fractions. *Enzyme and Microbial Technology* **15**:13-18.

Helle, S. S., Duff, S. J. B. and Cooper, D. G. 1993. Effect of Surfactants on Cellulose Hydrolysis. *Biotechnology and Bioengineering* **42**:611-617.

Heupel, C., Schlochtermeier, A and Schrempf, H. 1993. Characterization of an intracellular ß-Glucosidase from *Streptomyces reticuli. Enzyme and Microbial Technology* **15**:127-132.

Irwin, D., Spezio, M., Walker, L. P. and Wilson, D. B. 1993. Activity Studies of Eight Purified Cellulases: Specificity, Synergism, and Binding Domain Effects. *Biotechnology and Bioengineering* **42**:1002-1013.

Kanda, T., Wakabayashi, K. and Nisizawa, K. 1976a. Purification and Properties of an Endo-Cellulase of Avicelase Type from *Irpex lacteus (Polyporus tulipiferae). Journal of Biochemistry* **79**:977-988.

Kanda, T., Wakabayashi, K. and Nisizawa, K. 1976b. Xylanase Activity of an Endocellulase of Carboxymethyl-Cellulase Type from *Irpex lacteus (Polyporus tulipiferae). Journal of Biochemistry* **79**:989-995.

Kanda, T., Wakabayashi, K. and Nisizawa, K. 1976c. Synergistic Action of Two Different Types of Endo-Cellulase Components from *Irpex lacteus (Polyporus tulipiferae)* in the Hydrolysis of Some Insoluble Celluloses. *Journal of Biochemistry* **79**:997-1006.

Karunanandaa, K., Fales, S. L., Varga, G. A. and Royse, D. J. 1992. Chemical Composition and Biodegradability of Crop Residues Colonized by White-rot Fungi. *Journal of the Science of Food and Agriculture* **60**:105-112.

Kerem, Z., Friesem, D. and Hadar, Y. 1992. Lignocellulose Degradation during Solid-state Fermentation: *Pleurotus ostreatus* Versus *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **58**:1121-1127.

Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F. and Zeikus, J. G. 1978. Influence of Culture Parameters on Lignin Metabolism by *Phanerochaete chrysosporium. Archives of Microbiology* **117**: 277-285.

Kormelink, F. J. M. and Voragen, A. G. J. 1993. Degradation of Different [(Glucurono)arabino]xylans by a Combination of Purified Xylan-degrading Enzymes. *Applied Microbiology and Biotechnology* **38**:688-695.

Kormelink, F. J. M., Gruppen, H. and Voragen, A. G. J. 1993a. Mode of Action of (1,4)-ß-D-Arabinoxylan Arabinofuranohydrolase (AXH) and α-L-Arabinofuranosidases on Alkali-extractable Wheat-flour Arabinoxylan. *Carbohydrate Research* **249**:345-353.

Kormelink, F. J. M., Gruppen, H., Vietor, R. J. and Voragen, A. G. J. 1993b. Mode of Action of the Xylan-degrading Enzymes from *Aspergillus awamori* on Alkali-extratable Cereal Arabinoxylans. *Carbohydrate Reaseach* **249**:355-367.

Kubata, B. K., Suzuki, T., Horitsu, H., Kawai, K. and Takamizawa, K. 1993. Purification and Characterization of *Aeromonas caviae* ME-1 Xylanase V, Which Produces Exclusively Xylobiose from Xylan. *Applied and Environmental Microbiology* **60**:531-535.

Kubicek, C. P., Messner, R., Gruber, F., Mach, R. L. and Kubicek-Pranz, E. M. 1993. The *Trichoderma* Cellulase Regulatory Puzzle: From the Interior Life of a Secretory Fungus. *Enzyme and Microbial Technology* **15**:90-99.

Kuhad, R. C, and Singh, A.1993. Enhanced Production of Cellulases by *Penicillium citrinum* in Solid State Fermentation of Cellulosic Residue. *World Journal of Microbiology and Biotechnology* **9**:100-101.

Leatham, G. F. 1985. Extracellular Enzymes Produced by the Cultivated Mushroom *Lentinus edodes* during Degradation of a

Lignocellulosic Medium. *Applied and Environmental Microbiology* **50**:859-867.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry* **193**:265-275.

Lu, J, Y, and Tang, A. Y. 1986. Cellulolytic Enzymes and Antibacterial Activity of *Auricularia polytricha. Journal of Food Science* **51**:668-669.

MacKenzie, C. R., Bilous, D., Schneider, H. and Johnson, K. G. 1987. Induction of Cellulolytic and Xylanolytic Enzymes Systems in *Streptomyces* spp. *Applied and Environmental Microbiology* **53**:2835-2839.

Maheswari, D. K., Jahan, H., Paul, J. and Verma, A. 1993. Wheat Straw, a Potential Substrate for Cellulase Production Using *Trichoderma reesei. World Journal of Microbiology and Biotechnology* **9**:120-121.

Martin, S. and Akin, D. E. 1988. Effect of Phenolic Monomers on the Growth and ß-Glucosidase Activity of *Bacteroides ruminicola* and on the Carboxymethylcellulase, ß-Glucosidase, and Xylanase Activities of *Bacteroides succinogenes. Applied and Environmental Microbiology* **54**:3019-3022.

Masaphy, S. and Levanon, D. 1992. The Effect of Lignocellulose on Lignocellulolytic Activity of *Pleurotus pulmonarius* in Submerged Culture. *Applied Microbiology and Biotechnology* **36**:828-832.

McCarthy, A. J., Peace, E. and Broda, P. 1985. Studies on the Extracellular Xylanase Activity of Some Thermophilic Actinomycetes. *Applied Microbiology and Biotechnology* **21**:238-244.

Mishra, C. and Leatham, G. F. 1990. Recovery and Fractionation of the Extracellular Degradative Enzymes from *Lentinus edodes* Cultures Cultivated on a Solid Lignocellulosic Substrate. *Journal of Fermentation and Bioengineering* **69**:8-15.

Morawetz, R., Franz, G., Messner, R. and Kubicek, C. 1992. Presence, Transcription and Translation of Cellobiohydrolase Genes in Several *Trichoderma* species. *Current Genetics* **21**:31-36.

Moyson, E. and Verachtert, H. 1991. Growth of Higher Fungi on Wheat Straw and their Impact on the Digestibility of the Substrate. *Applied Microbiology and Biotechnology* **36**:421-424.

Nakanishi, K., Marui, M. and Yasui, T. 1992. Comparison of Xylan and Methyl ß-Xyloside-Induced Xylanases from *Streptomyces* sp. *Journal of Fermentation and Bioengineering* **74**:392-394.

Nidetzky, B. and Steiner, W. 1993. A New Approach for Modeling Cellulase-Cellulose Adsorption and the Kinetics of the Enzymatic Hydrolysis of Microcrystalline Cellulose. *Biotechnology and Bioengineering* **42**:469-479.

Nidetzky, B., Hayn, M., Macarron, R. and Steiner, W. 1993. Synergism of *Trichoderma reesei* Cellulases While Degrading Different Celluloses. *Biotechnology Letters* **15**:71-76.

Nidetzky, B., Zachariae, W., Gercken, G., Hayn, M. and Steiner, W. 1994. Hydrolysis of Cellooligosaccharides by *Trichoderma reesei* Cellobiohydrolases: Experimental Data and Kinetic Modeling. *Enzyme and Microbial Technology* **16**:43-52.

Norkrans, B. and Aschan, K. 1953. A Study in the Cellulolytic Variation for Wildtypes and Mutants of *Collybia velutipes.* II.- Relations to Different Nutrient Media. *Physiologia Plantarum* **6**:829-837.

Platt, M. W., Hadar, Y. and Chet, I. 1984. Fungal Activities Involved in Lignocellulose Degradation by *Pleurotus. Applied Microbiology and Biotechnology* **20**:150-154.

Poutanen, K. and Puls, J. 1988. Characteristics of *Trichoderma reesei* ß-Xylosidase and its Use in the Hydrolysis of Solubilized Xylans. *Applied Microbiology and Biotechnology* **28**:425-432.

Puls, J., Schmidt, D. and Granzow, C. 1987. α-Glucuronidase in Two Microbial Xylanolytic Systems. *Enzyme and Microbial Technology* **9**:83-88.

Rajarathnam, S., Wankhede, D. B. and Patwardhan, M. V. 1979. Some Chemical and Biochemical Changes in Straw Constituents During Growth of *Pleurotus flabellatus* (Berk and Br) Sacc. *European Journal of Applied Microbiology and Biotechnology* **8**:125-134.

Ramos, L. P., Breuil, C. and Saddler, J. N. 1993. The Use of Enzyme Recycling and the Influence of Sugar Accumulation on Cellulose Hydrolysis by *Trichoderma* Cellulases. *Enzyme and Microbial Technology* **15**:19-25.

Rao, M., Deshpande, V. and Mishra, C. 1985. Purification, Characterization, and Synergistic Action of Endoglucanases from *Fusarium lini. Biotechnology and Bioengineering* **28**:1100-1105.

Ratto, M., Poutanen, K. and Viikari, L. 1992. Production of Xylanolytic Enzymes by an Alkalitolerant *Bacillus circulans* Strain. *Applied and Environmental Microbiology* **37**:470-473.

Reese, E. T., Siu, R. G. H. and Levinson, H. S. 1950. The Biological Degradation of Soluble Cellulose Derivatives and its Relationship to the Mechanism of Cellulose Hydrolysis. *Journal of Bacteriology* **59**:485-497.

Richmond, P. A. 1991. Occurrence and Functions of Native Cellulose. In *Biosynthesis and Biodegradation of Cellulose.* (Haigler, C. H. and Weimer, P. J., eds.), Marcel Dekker, Inc., New York. pp.7.

Rothlisberger, P., Fiechter, A. and Zimmermann, W. 1992. Production of Thermostable Xylanases in Batch and Continuous Culture by *Thermomonospora fusca* KW3. *Applied Microbiology and Biotechnology* **37**:416-419.

Royer, J. C., Norak, J. S. and Nakas, J. P. 1992. Apparent Cellulase Activity of Purified Xylanase is due to Contamination of Assay Substrate with Xylan. *Journal of Industrial Microbiology* **11**:59-61.

Royse, D. J. 1992. Recycling of Spent Shiitake Substrate for Production of the Oyster Mushroom, *Pleurotus sajor-caju. Applied Microbiology and Biotechnology* **38**:179-182.

Schmidhalter, D. R. and Canevascini, G. 1992. Characterization of the Cellulolytic Enzyme System from the Brown-rot Fungus *Coniophora puteana. Applied Microbiology and Biotechnology* **37**:431-436.

Schmidt, O. and Dittberner, D. 1989. Biological and Enzymatic Variation in Strains of the Cultivated Mushroom Shii-take, *Lentinus edodes. Angewandte Botanik* **63**:55-65.

Somogyi, M. 1951. Notes on Sugar Determination. *Journal of Biological Chemistry* **195**:19-23.

Spear, L., Gallagher, L., McHale, L. and McHale, A. P. 1993. Production of Cellulase and ß-Glucosidase Activities Following Growth of

Streptomyces hygroscopicus on Cellulose Containing Media. *Biotechnology Letters* **15**:1265-1268.

Srivastava, R. and Srivastava, A. K. 1994. Characterization of a Bacterial Xylanase Resistant to Repression by Glucose and Xylose. *Biotechnology Letters* **15**:847-852.

Szakacs Dobozi, M., Szakacs, G. and Bruschi, C. V. 1992. Xylanase Activity of *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **58**:3466-3471.

Taj-Aldeen, S. J. 1993. Effect of Starch on the Induction of ß-Glucosidase in *Trichoderma reesei. Mycological Research* **97**: 318-320.

Taj-Aldeen, S. J. and Alkenany, K. I. 1993. Properties of the Cellulolytic System from *Aspergillus niveus. Mycological Research* **97**:15-22.

Tarchevsky, I. A. and Marchenko, G. N. 1991. *Cellulose: Biosynthesis and Structure,* Springer-Verlag, Germany. pp.1.

Teunissen, M., De Kort, G. V. M., Op Den Camp, H. J. M. and Huis In't Veld, J. H. J. 1992. Production of Cellulolytic and Xylanolytic Enzymes During Growth of the Anaerobic Fungus *Piromyces* sp. on Different Substrates. *Journal of General Microbiology* **138**:1657-1664.

Tilbeurgh, H., Bhikkabhai, R., Pettersson, G. and Claeyssens, M. 1984. Separation of Endo- and Exo-type Cellulases Using a New Affinity Chromatography Method. *FEBS Letters* **169**:215-218.

Tilbeurgh, H., Loontiens, F. G., Engelborgs, Y. and Claeyssens, M. 1989. Studies of the Cellulolytic System of *Trichoderma reesei* QM9441 - Binding of Small Ligands to the 1,4-ß-Glucan Cellobiohydrolase II and Influence of Glucose on their Affinity. *European Journal of Biochemistry* **184**:553-559.

Tsang, L. J., Reid, I. D. and Coxworth, E, C. 1987. Delignification of Wheat Straw by *Pleurotus* spp. under Mushroom-growing Conditions. *Applied and Environmental Microbiology* **53**:1304-1306.

Uchida, H., Kusakabe, I., Kawabata, Y., Ono, T. and Murakami, K. 1992. Production of Xylose from Xylan with Intracellular Enzyme System of *Aspergillus niger* 5-16. *Journal of Fermentation and Bioengineering* **74**:153-158.

Uzcategui, E., Ruiz, A., Montesino, R., Johansson, G. and Pettersson, G. 1991. The 1,4-ß-D-Glucan Cellobiohydrolases from *Phanerochaete chrysosporium*. I. A System of Synergistically Acting Enzymes Homologous to *Trichoderma reesei*. *Journal of Biotechnology* **19**:271-286.

Vian, B. and Reis, D. 1991. Relationships of Cellulose and Other Cell Wall Components: Supramolecular Organization. In *Biosynthesis and Biodegradation of Cellulose.* (Haigler, C. H. and Weimer, P. J., eds.), Marcel Dekker, Inc., New York. pp.26.

Walker, L. P., Wilson, D. B., Irwin, D. C., McQuire, C. and Price, M. 1992. Fragmentation of Cellulose by the Major *Thermomonospora fusca* Cellulases, *Trichoderma reesei* CBHI, and Their Mixtures. *Biotechnology and Bioengineering* **40**:1019-1026.

Wang, W. K., Kruus, K. and Wu, J. H. D. 1993. Cloning and DNA Sequence of the Gene Coding for *Clostridium thermocellum* Cellulase S_2 (CelS), a Major Cellulosome Component. *Journal of Bacteriology* **175**:1293-1302.

Westermark, U. and Eriksson, K. E. 1974. Cellobiose:Quinone Oxidoreductase, a New Wod-degrading Enzyme from White-rot Fingi. *Acta Chemica Scandinavica B* **28**:209-214.

Wood, D. A. and Smith, J. F. 1987. The Cultivation of Mushrooms. In *Essays in Agricultural and Food Microbiology* (Norris, I. R. and Pettipher, G. L., eds.), John Wiley and Sons Ltd., New York. pp.332-333.

Wood, D. A., Claydon, N., Dudley, K. J., Stephens, S. K. and Allan, M. 1988. Cellulase Production in the Life Cycle of the Cultivated Mushroom, *Agaricus bisporus.* In *Biochemistry and Genetics of Cellulose Degradation* (Aubert, J. P., Beguin, P. and Millet, J., eds.), Academic Press, London. pp.53-70.

Wood, T. M. 1991. Fungal Cellulases. In *Biosynthesis and Biodegradation of Cellulose* (Haigler, C. H. and Weimer, P. J., eds.), Marcel Dekker, Inc., New York.

Wood, T. M. and Bhat, K. M. 1988. Methods for Measuring Cellulase Activities. In *Methods in Enzymology, Volume 160, Biomass Part A, Cellulose and Hemicellulose* (Wood, W. A. and Kellogg, S. T., eds.), Academic Press, Inc., San Diego, California. pp.87-112.

Wood, T. M. and McCrae, S. I. 1972. The Purification and Properties of the C_1 Component of *Trichoderma koningii* Cellulase. *Biochemical Journal* **128**:1183-1192.

Wood, T. M. and McCrae, S. I. 1978. The Cellulase of *Trichoderma koningii. Biochemical Journal* **171**:61-72.

Wood, T. M. and McCrae, S. I. 1986. The Cellulase of *Penicillium pinophilum* - Synergism between Enzyme Components in Solubilizing Cellulose with Special Reference to the Involvement of Two Immunologically Distinct Cellobiohydrolases. *Biochemical Journal* **234**:93-99.

Wood, T. M. and McCrae, S. I. and Bhat, K. M. 1989. The Mechanism of Fungal Cellulase Action. *Biochemical Journal* **260**:37-43.

Woodward, J., Lima, M. and Lee, N. 1988. The Role of Cellulase Concentration in Determining the Degree of Synergism in the Hydrolysis of Microcrystalline Cellulose. *Biochemical Journal* **255**:895-899.

Yague, E., Beguin, P. and Aubert, J. P. 1990. Nucleotide Sequence and Deletion Analysis of the Cellulase-encoding Gene *cel*H of *Clostridium thermocellum. Gene* **89**:61-67.